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The goal of this award was to identify cytoplasmic proteins that associate with the cytoplasmic tail of PSMA. Using yeast tow-hybrid screening, we identified that PSMA associates with an actin cross-linkin proteins called filamin. We have demonstrated this interaction both in vitro and in vivo. We also established that PSMA is localized to he recycling endosome and that PSMA association with filamin is necessary for PSMA targeting to the recycling endosome. Finally, we also showed that the cytoplasmic tail of PSMA through its association with the actin cross-linking protein modulae the NAALADase activity of this protein. We suggest that the cytoplasmic tail of PSMA plays an important role in the function/s of PSMA.

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## Progress report

**Introduction:** Prostate specific membrane antigen (PSMA) is the single most well established, highly restricted prostate epithelial cell membrane antigen. In contrast to other highly restricted prostate-related antigens such as prostate specific antigen (PSA) and prostatic acid phosphatase (PAP), which are secretory proteins, PSMA is an integral membrane protein. In addition to its prostate specificity, PSMA is expressed by a very high proportion of prostate cancers (PCa). Expression is further increased in higher-grade cancers, in metastatic disease, and in hormone- refractory PCa. Why PSMA expression is increased in prostate cancer cells and whether PSMA has a role in prostate cancer development or progression is not known. Understanding the function of PSMA should provide insights into the role of PSMA in normal prostate as well as in prostate cancer. Our recent results indicate that PSMA is a multifunctional protein with distinct functions associated with the extracellular and cytoplasmic domains. The extracellular domain of PSMA has glutaminase activity including folate hydrolase and NAALADase activities. We now found that the cytoplasmic tail of PSMA is involved in its internalization and targeting to recycling endosomal compartment (REC). In this proposal we sought to characterize the mechanism by which the cytoplasmic tail of PSMA mediates this function. We have completed this proposal very successfully and have published three important papers in 2003.

**Body:** We have shown that PSMA is constitutively internalized in LNCaP cells (human prostate cancer cell line) and suggested that PSMA may have the function of a receptor internalizing a putative ligand (Liu et al., 1998). Using a variety of cell and molecular biology techniques we have now characterized a novel methionine-leucine based motif in the cytoplasmic tail of PSMA as its internalization and lysosomal targeting signal (Rajasekaran et al., manuscript enclosed in Appendix). Since a well-defined normal prostate epithelial cell line is not available, to understand more about the function of PSMA, we expressed PSMA in Madin-Darby Canine Kidney (MDCK) cells that are widely being used as an epithelial model. Cultured MDCK cells have distinct apical and basolateral plasma membrane domains like epithelial tissue in vivo. First, we characterized that MDCK cells are appropriate model to study the function of PSMA. For this purpose, we compared the localization of PSMA expressed in MDCK cells and in normal prostate. We found that PSMA is localized to the apical plasma membrane in normal prostate as well as in MDCK cells. This result indicated that protein targeting machinery for PSMA in MDCK cells and prostate tissue is conserved and that MDCK cells could be used as a model to study PSMA function (Christiansen et al., 2003, reprint enclosed in Appendix).

We have now characterized that localization of PSMA to REC requires its association with Filamin (FLNa). We also showed that FLNa association with PSMA modulates its NAALAdase activity (Anilkumar et al., 2003, reprint enclosed in Appendix). FLNa is a high molecular weight actin cross-linking phosphoprotein and is an important structural determinant of the submembranous cytoskeletal structure in animal cells (Gorlin et al., 1990). It serves as docking site for various cell surface receptors and intracellular proteins involved in signal transduction and localizes to structures involved in cell motility such as filopodia (Ohta et al., 1999). Our results for the first time reveal that filamin is an important regulator of PSMA function. These results also contributed a key finding on the organization of recycling endosomal compartment (REC). REC plays an important role in the recycling of various

membrane proteins such as transferrin receptor, insulin receptor, and epidermal growth factor receptor. Although much is known about the mechanism of recycling of these receptors less is known about the mechanisms involved in the organization of REC and how proteins are targeted to the REC. Our findings suggest that filamin is involved in both organization and targeting proteins to the REC. In addition, our results also suggest that PSMA is a marker for REC and recycles through REC like transferrin receptor and other receptors implicating that PSMA might function as a putative receptor for a ligand yet to be identified. During these studies we also found that LNCaP cells do not have an organized REC. Currently, experiments are in progress to demonstrate the absence of REC in this cell line and its consequence on receptor recycling. This observation is crucial since absence of organized REC in this cell line might have altered the kinetics of recycling of receptors as described above that recycle through the REC. The implications of these findings are discussed in detail in the enclosed manuscript (Anilkumar et al., 2003). These findings further led us that targeting to recycling endosome might be mediated by specific signals in the cytoplasmic tail of PSMA. Consistent with this hypothesis we have now identified a novel methionine-leucine based internalization motif designated as MXXXL in the cytoplasmic tail of PSMA. We expect that filamin might be binding to this motif and mediate targeting of PSMA to REC. These studies for the first time revealed that targeting to REC might be signal mediated and suggested a possible role for filamin in this function. Experiments are in progress in our laboratory to further characterize the significance of PSMA association with filamin.

**Task 1. Prepare PSMA cytoplasmic tail bait, yeast two-hybrid screening, and COLT (months 1-24)**

- Prepare λgt11 libraries from RNA isolated from MDCK and LNCaP cells
- Start yeast two-hybrid screening of both MDCK and LNCaP libraries and identify positive clones by retransformation with bait
- Start COLT in both MDCK and LNCaP cell λgt11 libraries and purify positive plaques
- Sequence clones obtained from yeast two-hybrid screening and COLT
- If novel proteins are identified start preparation of polyclonal peptide antibodies
- If known proteins are identified use available antibodies to test co-distribution, co-immunoprecipitation, and co-sedimentation with PSMA

**Progress:** Using PSMA cytoplasmic tail as bait for yeast two-hybrid screening we have identified FLNa as a binding partner and have characterized this interaction both in vivo and in vitro. We have now demonstrated that PSMA is localized to REC and that FLNa association is necessary for targeting PSMA to the REC. We have also shown that PSMA NAALAdase activity is regulated by filamin. These studies have now determined that filamin is an important regulator of PSMA function.

**Task 2. Develop the *in vitro* phosphorylation assay (months 12-36)**

- Start making cytoplasmic tail phosphorylation mutants
- Generate stable clones expressing phosphorylation mutants
- Test localization of PSMA in MDCK cells expressing phosphorylation mutants

. Analyze the role of PSMA phosphorylation mutants in cell cycle using FACS.

**Progress:** A PSMA mutant in which all the phosphorylation sites have been mutated has been made. This construct is clearly localized to the recycling endosomal compartment. Further mutagenesis studies have determined that the first five aminoacids of PSMA is important for its localization to the recycling endosome

**Key Research Accomplishments:**

- ✓ Established MDCK cells as a model to study targeting prostate restricted proteins.
- ✓ Identified a potential role of PSMA in cell cycle regulation.
- ✓ Identified Filamin as a cytoplasmic tail binding protein of PSMA using yeast two hybrid analysis
- ✓ Characterized binding of filamin to PSMA cytoplasmic tail both by in vitro and in vivo methods
- ✓ Characterized that PSMA filamin association is necessary for pericentrosomal targeting of PSMA
- ✓ Determined that PSMA filamin association modulates NAALADase activity of PSMA
- ✓ Determined that filamin might play a role in the organization of recycling endosome
- ✓ Determined that LNCaP cells do not have an organized recycling endosome.
- ✓ Phosphorylation of PSMA is not necessary for its localization to the recycling endosomal compartment.
- ✓ Identification of a novel signal to target proteins to the recycling endosomal compartment.

**Reportable outcomes**

**Published papers (2003)**

1. Christiansen, J., S.A. Rajasekaran, P. Moy, A. Butch, L. Goodglick, N. Bander, R.Reiter, Z. Gu and A.K. Rajasekaran (2003). Polarity of Prostate specific membrane antigen, Prostate stem cell antigen, and prostate specific antigen in MDCK cells and in prostate tissue (Prostate, 55:9-19).
2. Rajasekaran, S.A., G. Anilkumar, E.Oshima, J.Bowie, H. Liu, W.Heston, N.H. Bander, A. K Rajasekaran (2003). A novel cytoplasmic tail MXXXL motif mediates the internalization of prostate specific membrane antigen (Mol.Biol.Cell.14:4835-4845).
3. Anilkumar, G., S.A.Rajasekaran, S.Wang, O.Hankinson, N. H Bander, and A. K Rajasekaran (2003). Prostate specific membrane antigen association with filamin A modulates its internalization and NAALADase activity (Cancer Res. 63: 2645-2648).

**Presentations at the conferences: (Abstracts)**

1. Eri Oshima, Sigrid A Rajasekaran, Geoffrey Kim, Ning Ru, He Liu, Warren Heston, Neil H Bander, Ayyappan K Rajasekaran (2001). A cytoplasmic tail di-

leucine motif mediates the internalization and lysosomal targeting of prostate specific membrane antigen. (New discoveries in prostate cancer, AACR meeting, Naples, Florida).

2. Christiansen, J., S.A. Rajasekaran, B. Krishnamachary, P. Moy, A. Butch, L. Goodglick, N. Bander, A.K. Rajasekaran (2002). MDCK cells as model system to study polarity of prostate restricted proteins (Abstract #107227, 93rd Annual meeting of AACR, San Francisco, CA).

3. Rajasekaran, S.A., G. Anilkumar, E. Oshima., G. Kim, N. Ru, H. Liu, W. Heston, N. Bander, and A.K Rajasekaran (2002). A cytoplasmic tail leucine-based motif mediates the internalization of prostate specific membrane antigen. (American Society for Cell Biology Annual Meeting, San Francisco, CA).

**Conclusions:** We have characterized MDCK cells as a potential model to study targeting of prostate specific proteins. We have identified a novel methionine-leucine based motif in the cytoplasmic tail of PSMA as its internalization and REC targeting signal. We have also shown that the cytoplasmic tail of PSMA association with FLNa is necessary for its targeting to the REC. We have also shown that PSMA-filamin association modulates the NAALADase activity of PSMA.

**References:**

Liu H, Rajasekaran, A, Moy, P., Xia, Y., Kim, S, Navarro, V., Rahmati, R, and. Bander N. 1998. Constitutive and antibody induced internalization of prostate specific membrane antigen. Cancer Res. 58: 4055-4060.

Ohta, Y., N. Suzuki, S. Nakamura, J.H. Hartwig, and T.P. Stossel. 1999. The small GTPase RalA targets filamin to induce filopodia. *Proc Natl Acad Sci U S A*. 96:2122-8.

Gorlin, J.B., R. Yamin, S. Egan, M. Stewart, T.P. Stossel, D.J. Kwiatkowski, and J.H. Hartwig. 1990. Human endothelial actin-binding protein (ABP-280, nonmuscle filamin): a molecular leaf spring. *J Cell Biol.* 111:1089-105.

**Appendix:** 1.Three reprints.

# Polarity of Prostate Specific Membrane Antigen, Prostate Stem Cell Antigen, and Prostate Specific Antigen in ProstateTissue and in a Cultured Epithelial Cell Line

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**BACKGROUND.** Madin-Darby canine kidney (MDCK) cells are immortalized epithelial cells that have been used extensively as a model system to study intracellular molecular trafficking, polarized expression, and secretion of proteins in various epithelia. In order to determine if MDCK cells might serve as a model to study molecular events within prostate epithelial cells, we have evaluated the polarized distribution of three prostate restricted proteins, PSMA, PSCA, and PSA, in situ, and in MDCK cells.

**METHODS.** Using immunofluorescence, confocal microscopy, cell surface biotinylation, antibody internalization, and biochemical assays we evaluated surface expression and secretion of three prostate restricted proteins expressed in MDCK cells. We compared these patterns of expression to results observed within prostatic epithelium.

**RESULTS.** We demonstrate that PSMA is localized primarily to the apical plasma membrane in both the prostatic epithelium and transfected MDCK cells, whereas PSCA is expressed in a non-polarized fashion. We also show that PSA is secreted predominantly from the apical surface of transfected MDCK cells, consistent with in vivo observations.

**CONCLUSIONS.** Similar patterns of localization among MDCK and prostatic epithelial cells suggest that the mechanisms of polarized sorting within these cell types are conserved. Thus, MDCK cells offer a useful model system to study mechanisms of targeting of these proteins within the prostate. *Prostate 55: 9–19, 2003.* © 2003 Wiley-Liss, Inc.

*KEY WORDS:* **prostate; prostate neoplasm; prostate specific membrane antigen; prostate stem cell antigen; prostate specific antigen; protein targeting**

## INTRODUCTION

External surfaces and internal cavities of the body are lined with a thin layer of epithelial tissue. These tissues are comprised of polarized epithelial cells in close apposition to one another and adherent upon a thin, non-cellular basement membrane attached to the underlying connective tissue. Epithelial tissues functionally partition biological compartments, physically separating them from one another or from the external environment. These tissues form selective permeability barriers between compartments, and allow for

Abbreviations used: PSMA, prostate specific membrane antigen; MDCK, Madin-Darby canine kidney cells; PSCA, prostate stem cell antigen; PSA, prostate specific antigen.

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vectorial transport of fluids, ions, proteins, and other solutes, thus enabling organs to perform vital physiological functions, such as secretion, absorption, ion transport, or formation of impervious fluid barriers [1].

The ability of epithelia to perform these specialized feats of vectorial transport resides in the unique cellular architecture of the polarized epithelial cell. These cells are composed of two biochemically distinct plasma membrane surfaces, each with a unique composition of lipids and proteins. The apical plasma membrane, which faces the lumenal or extracellular space, is physically separated from the basolateral membrane, which is in contact with adjacent cells or the underlying matrix, by the zonula occludens, or tight junctions. The tight junctions completely circumscribe the cell just below the apical surface, creating regions of tight membrane contact between adjacent cells [2]. The tight junctions prevent lateral diffusion of molecules between plasma membrane domains, as well as restrict the flow of fluid through intercellular spaces.

The glandular epithelium of the prostate is composed of specialized secretory epithelial cells that produce and secrete a portion of the seminal fluid. Recent efforts have led to the identification of several proteins with expression highly restricted to the prostatic epithelium, including prostate specific membrane antigen (PSMA) [3], prostate stem cell antigen (PSCA) [4], prostate specific antigen (PSA) [5], prostatic acid phosphatase (PAP) [6], and six transmembrane epithelial antigen of the prostate (STEAP) [7].

Originally identified in LNCaP cells, PSMA is a type II transmembrane glycoprotein of approximately 100 kDa [3]. Although the precise physiological role of PSMA is not known, the large extracellular domain possesses glutamate carboxypeptidase [8] and folate hydrolyase [9] activities, thus PSMA has been implicated in the generation of glutamate by hydrolyzing peptide substrates within prostatic fluid [8]. In addition to expression in the normal prostate, PSMA expression is increased in virtually all cases of prostate cancer, and is further upregulated in high grade tumors and androgen independent disease [10–12]. Recent findings also indicate that PSMA is selectively expressed in the neovasculature of nearly all types of solid tumors, but not in the vasculature of normal tissue [10,13–15]. There is currently a great deal of clinical interest in PSMA for diagnostic purposes, in vivo imaging strategies, and cancer therapies [16].

Expression of PSCA is also highly restricted to the prostatic epithelium, with limited expression observed in some extraprostatic tissues. PSCA is a homologue of the Thy-1/Ly-6 family of glycosylphosphatidylinositol (GPI)-anchored proteins [4]. PSCA is overexpressed in a high proportion of localized and metastatic prostate tumors, and levels of expression have been determined to correlate with tumor stage, grade, and androgen independence [17]. For this reason, the prospect of exploiting PSCA as a prognostic indicator and as a target for immunotherapy are being explored [18,19].

PSA has proven one of the most useful biological markers in the diagnosis and management of prostate cancer [20,21]. PSA is a kallikrein-like serine protease that is secreted at high levels from the apical surface of the prostatic epithelium into the lumen of the gland [22,23]. PSA is one of the most abundant proteases found in prostatic fluid [24], and is believed to enhance sperm motility by participating in the liquification of semen by degrading matrix proteins [25,26]. PSA is also found in the serum, with increased levels associated with prostate cancer [27]. This may suggest that while PSA is secreted predominantly from the apical surface into the glandular lumen, a lower level of PSA is secreted from the basolateral surface, where it can gain access to the vasculature.

Despite the prevalence of prostate cancer and other pathological conditions, such as benign prostatic hyperplasia (BPH) and prostatitis, there currently exists no described cell culture model system to study polarized sorting of proteins within the prostatic epithelium. The common prostate derived cell lines, such as LNCaP, PC3, and DU145 are unsuitable for this purpose, as they are highly transformed, non-polarized cells that fail to form tight junctions. The lack of an appropriate model system in which to study polarized sorting of proteins within the prostatic epithelium has led us to investigate the potential of using Madin-Darby canine kidney (MDCK) cells for this purpose. MDCK cells are a well-differentiated cell line with tight junctions that form polarized monolayers in culture that closely resemble epithelial tissue. MDCK cells have previously been used as a model system to study epithelial polarity within a variety of organs, including the liver [28,29], kidney [30], thymus [31], thyroid [32], and intestine [33,34]. We have expressed three prostate restricted proteins, PSMA, PSCA, and PSA, in MDCK cells and compared the pattern of expression to that observed within the prostate tissue. In this manuscript, we demonstrate that like in prostate tissue PSMA is localized predominantly to the apical plasma membrane in MDCK cells. Furthermore, we demonstrate that PSCA is expressed in a non-polarized fashion in both prostate tissue and in transfected MDCK cells. Additionally, we show that PSA, which is primarily secreted into the lumen of the prostate gland, is secreted predominantly from the apical side in MDCK cells. These studies for the first time provide evidence that MDCK cells can be utilized as model to study polarized sorting of prostate restricted proteins and suggest that these cells could be utilized to further understand the function/s of these proteins.

## MATERIALS AND METHODS

### Prostate Tissue

Remnant prostate tissue specimens were obtained from the Human Tissue Research Center at UCLA with proper approval by the UCLA office for the protection of research subjects. Following surgical removal, remnant specimens were rapidly placed in OCT compound and snap frozen using dry ice and 2-methyl butane mixture. Frozen sections (5 μm) were prepared and utilized for immunofluorescence studies. Pathologists at UCLA evaluated all prostate tissues. Tumor and matched normal tissues from a total of 12 patients diagnosed with prostate cancer (five patients with Gleason scores of 6, six with scores of 7, and one with score of 9) were used for immunofluorescence studies.

### Cell Culture

MDCK cells (clone II) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 25 U/ml penicillin, 25 μg/ml streptomycin, and 100 μM non-essential amino acids. Cells were grown at 37°C in a humidified incubator with 5% $CO_2$. MDCK cell lines expressing PSMA were treated overnight in media containing 10 mM sodium butyrate to increase PSMA expression.

### DNA Constructs and Transfections

The cDNAs encoding full length PSMA (kindly provided by Dr. Warren Heston, Cleveland Clinic Foundation, OH) or PSCA were cloned into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA). The pSecTag2/PSA vector containing the cDNA encoding the active form of PSA fused to a *myc* epitope and polyhistidine tag was purchased from Invitrogen.

To generate stable cell lines, MDCK cells were transfected using the calcium phosphate method described previously [35]. Clones were selected in the presence of 500 μg/ml geneticin (G418, Gibco BRL, Rockville, MD) for pcDNA3 vectors or 300 μg/ml Zeocin (Invitrogen) for the pSecTag2/PSA vector. Expression was confirmed by immunofluorescence and immunoblotting.

Transient transfection was performed using lipofectamine reagent (Gibco BRL) according to manufacturer's instructions.

### Antibodies

The monoclonal antibody (mAb) J591 directed against an extracellular epitope of PSMA has previously been described [15]. The mAb 7E11 directed against PSMA was prepared from hybridoma 7E11 (ATCC, Rockville, MD). The mAb to GP-135 was generously provided by Dr. Ojakian (State University of New York, NY). Generation of the mAb 1G8 against PSCA was previously described [17]. Antibody to E-cadherin was purchased from Zymed (South San Fransisco, CA). Antibody to β-catenin and HRP conjugated goat-anti-mouse IgG were purchased from Transduction Laboratories (Lexington, KY). FITC conjugated goat-anti-mouse IgG, Texas Red conjugated goat-anti-mouse IgG, and CY3 conjugated donkey-anti-mouse IgG secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

### Immunofluorescence

Tissue sections were fixed in cold methanol at −20°C for 30 min. Following fixation, specimens were placed in humidified chambers, washed with phosphate buffered saline containing 100 μM $CaCl_2$, 1 mM $MgCl_2$, and 0.5% bovine serum albumin (PBS-CM-BSA), incubated 1 hr with primary antibody, washed with PBS-CM-BSA, incubated for 30 min in secondary antibody, washed with PBS-CM-BSA, and rinsed with distilled water. Propidium iodide (1 μg/ml) was added after incubation with secondary antibody and RNaseA (5 μg/ml) and cells were washed twice with PBS-CM-BSA. Specimens were mounted in vectashield (Vector, Burlingame, CA) and the coverslips were sealed.

Two forms of fixation protocol were used for MDCK cells, cold methanol fixation, which renders the cells permeabilized, and paraformaldehyde (PFA) fixation, which does not permeabilize cells. Cold methanol fixation of MDCK cells was performed as described above for tissues. For non-permeablized fixation, MDCK cells grown on glass coverslips were incubated in the dark for 30 min in a 2% solution of PFA with 100 μM $CaCl_2$, and 1 mM $MgCl_2$. Coverslips were washed twice in PBS-CM-BSA and incubated with 50 mM $NH_4Cl$ in PBS-CM for 10 min and further processed similar to tissues. For cell surface staining, MDCK cells expressing PSMA were grown to confluency on glass coverslips. Media was removed and replaced with chilled DMEM containing 10 μg/ml J591. Cells were incubated on ice for 1 hr, rinsed with cold PBS-CM-BSA, fixed in cold methanol, and incubated with secondary antibody as described above.

For surface staining of MDCK cells expressing PSCA, confluent monolayers were grown on polycarbonate transwell filters (Corning, New York). Cells were stained for 30 min with 10 μg/ml of 1G8 in PBS (2% FCS) added on ice to both the apical and basolateral chambers. After removal of unbound antibody, cells were fixed in cold methanol and incubated with secondary antibody as described above.

### Confocal Microscopy

Confocal microscopy was performed using a Fluoview laser scanning confocal microscope (Olympus America, Melville, NY) as described previously [36]. To detect FITC-labeled antigens and propidium iodide, samples were excited at 488 nm with an argon laser and light emitted between 525 and 540 nm was recorded for FITC and above 630 nm for propidium iodide. Images were generated and analyzed using the Fluoview image analysis software (version 2.1.39).

### Antibody Uptake

MDCK cells expressing PSMA were grown to confluency on glass coverslips. Media was removed and replaced with 1 ml of DMEM containing 10 μg/ml J591. Cells were incubated at 37°C for 3 hr, rinsed in PBS-CM-BSA, fixed in cold methanol, and incubated with secondary antibody as described above.

### Transepithelial Electrical Resistance (TER) Measurements

The resistance of MDCK monolayers on polycarbonate transwell filters (Corning) was determined using an EVOM Epithelial Voltometer (World Precision Instruments, Sarasota, FL). Values were normalized for the area of the filter after subtracting the background resistance of a filter without cells. TER of 220–250 $\Omega cm^2$ is indicative of the presence of functional tight junctions [37].

### Cell Surface Biotinylation

MDCK cells expressing PSMA were grown on transwell filters. Tight junction formation was assessed by TER. Biotinylation of apical or basolateral surfaces was performed as previously described [35]. Briefly, the cell surface of confluent monolayers was labeled on ice from apical or basolateral surface with 0.5 μg/ml of membrane impermeable EZ-Link Sulfo-NHS-Biotin (Pierce, Rockford, IL) in TEA (150 mM NaCl, 10 mM Triethanolamide pH 9.0, 1 mM $CaCl_2$, 1 mM $MgCl_2$). After quenching (50 mM $NH_4Cl$ in PBS-CM), the cells were lysed in 0.5 ml lysis buffer (150 mM NaCl, 20 mM Tris pH8, 5 mM EDTA, 1% Triton-X-100, 0.1% BSA, 1 mM PMSF, 5 μg/ml each of antipain, leupeptin, and pepstatin). Total protein for each lysate was used for precipitation (16 hr at 4°C) with mAb J591 bound to protein A sepharose beads. Precipitates were washed, separated on a 10% SDS polyacrylamide gel, transferred to nitrocellulose, immunoblotted with the streptavidin-HRP, and visualized by ECL as described earlier [38].

### PSA Assay

MDCK cells expressing PSA were grown on transwell filters. Tight junction formation was assessed by TER. Two millilitres of fresh media was added to both the apical and basolateral chambers, cells were incubated 8–10 hr at 37°C, and media was collected from each chamber.

PSA concentrations in tissue culture media were determined using the automated Access Immunoassay Analyzer (Beckman Coulter, Fullerton, CA) using the Hybritech PSA 1-Step sandwich assay. The assay measures both free and complexed PSA on an equal molar basis. Recovery experiments were performed to verify that the assay accurately measures PSA in tissue culture medium by adding known quantities of PSA to samples of DMEM tissue culture medium. Total imprecision (coefficient of variation) for the assay at PSA concentrations of 2.7 and 21.5 ng/ml were 4.6 and 3.2%, respectively.

## RESULTS

Frozen sections from regions of tumor and morphologically normal tissue from 12 patients were stained for PSMA and subject to analysis. In matched tissue samples from 10 of the 12 patients, confocal microscopy revealed a prominent PSMA staining along the lumenal surface (apical) of the prostate gland in tumor tissues (Fig. 1A). Although apically localized, the staining intensity of PSMA in normal tissues was relatively weak (data not shown). In these tissues, the basolateral marker protein E-cadherin was distinctly localized to the basolateral plasma membrane (Fig. 1B). These results indicated that PSMA is predominantly localized to the apical plasma membrane in prostate gland. Tumor tissue sections from two patients (Gleason scores 6 and 7) with high levels of PSMA expression showed a distinctly non-polarized distribution of PSMA with staining on both apical and basolateral plasma membranes (Fig. 1C, arrow). Interestingly, E-cadherin in the same gland showed polarized localization to the basolateral plasma membrane (Fig. 1D).

To study the localization of PSMA in MDCK cells, we generated a stable cell line expressing PSMA (MDCK-PSMA). These cells produced a glycoprotein that migrated through an SDS polyacrylamide gel with an apparent molecular mass of 100 kDa and was recognized by the mAb, 7E11. Following treatment with *N*-glycosidase, the core, deglycosylated protein ran with a molecular mass of approximately 84 kDa. This is in agreement with observations in LNCaP cells, which express high levels of endogenous PSMA, suggesting that the transfected MDCK cell line is expressing a full-length and glycosylated form of PSMA (Fig. 2).

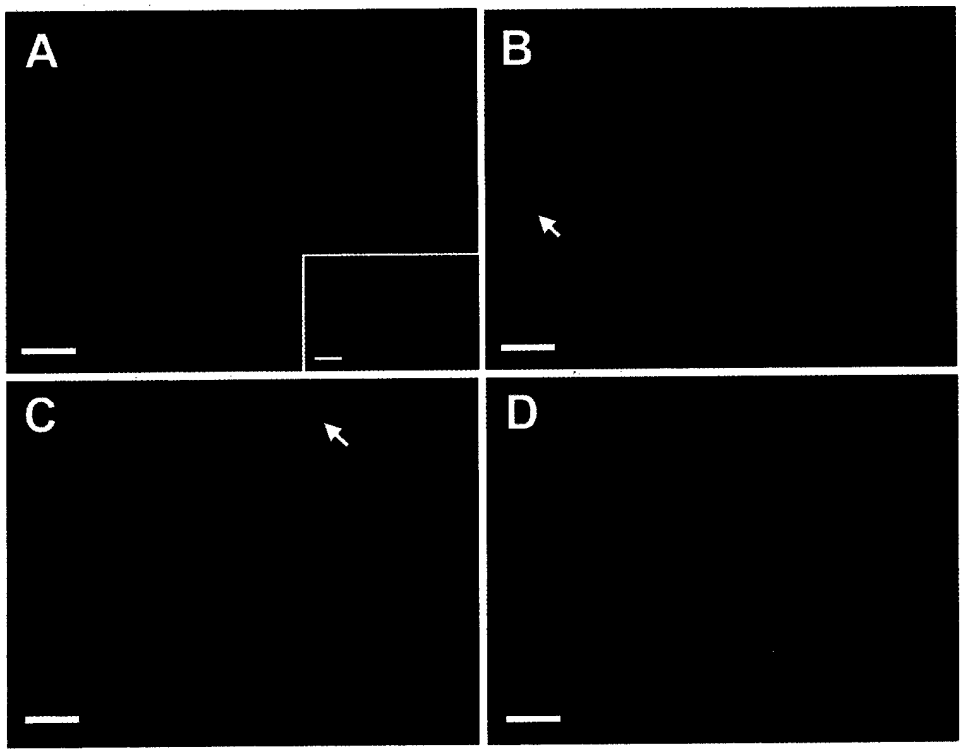


**Fig. 1.** Localization of PSMA and E-cadherin in prostate tissue. Tumor tissue sections stained with mAb 7E11 and FITC-conjugated secondary antibody (green) reveal an apical staining pattern for PSMA (**A**). No staining is visible after incubation with secondary antibody alone (A inset). Basolateral staining is evident when tissue sections are stained with mAb directed against E-cadherin and FITC-conjugated secondary antibody (**B**). Basolateral localization of PSMA (**C**, arrow) and E-cadherin in the same prostate gland (**D**). Propidium iodide was used to stain nuclei in all panels. Bars, 15 μm.

To determine the plasma membrane localization of PSMA in MDCK cells, confluent monolayers of MDCK-PSMA cells were grown on glass coverslips. Surface immunofluorescence and confocal microscopy analyses revealed an apical staining pattern of PSMA. The staining pattern of PSMA in horizontal (XY) and vertical (XZ) confocal microscope optical sections (Fig. 3A) was similar to that of an endogenously expressed apical marker GP-135 (Fig. 3B) in MDCK cells. The apical localization of PSMA and GP-135 can be contrasted to the typical basolateral staining pattern of β-catenin (Fig. 3C).

To further evince PSMA expression on the apical plasma membrane, we utilized an antibody internalization assay [38]. In this assay, confluent monolayers MDCK-PSMA cells were incubated with mAb J591. Tight junction prevents the mAb J591 from reaching antigens localized to the basolateral plasma membrane. Therefore, internalization of antibody will occur only from the apical plasma membrane. The vesicular endosomal staining shown in Figure 4E, demonstrates that mAb J591 was clearly internalized from the apical surface in MDCK-PSMA cells. To confirm that these monolayers contain functional tight junctions and are not permeable to antibody, we utilized an antibody against the extracellular domain of E-cadherin, a basolateral marker in MDCK cells. When cells were permeablized, E-cadherin was visualized on the basolateral plasma membrane (Fig. 4A and B). Under non-permeablized conditions, incubation of the MDCK-PSMA monolayer with E-cadherin antibody did not show staining of the basolateral plasma membrane (Fig. 4C and D).

To obtain a quantitative analysis of PSMA expression on the cell surface, we performed cell surface biotinylation experiments. Identical numbers of MDCK-PSMA cells were grown on transwell filters, and formation of tight junctions was confirmed by measuring TER. Biotinylation of either apical or basolateral surfaces was performed, cells were lysed, and the levels of biotinylated PSMA on either surface were determined (Fig. 5A). The results indicated that of the PSMA expressed on the cell surface, 70% was localized to the apical plasma membrane whereas about 30% was localized to the basolateral plasma membrane (Fig. 5B). Taken together, these results strongly suggest

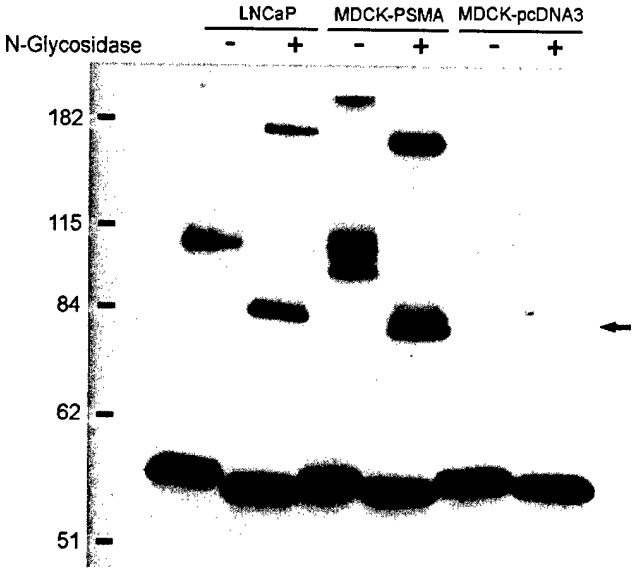


**Fig. 2.** PSMA expression in LNCaP and MDCK cells. PSMA was immunoprecipitated from LNCaP or MDCK cells using mAb J591. The precipitated samples were treated for 4 hr at 37°C in the presence or absence of N-glycosidase. Western blot analysis using mAb 7E11 reveals a glycoprotein of approximately 100 kDa and a core deglycosylated protein of approximately 84 kDa (arrow) in both LNCaP and MDCK-PSMA cells. No bands are recognized by 7E11 in untransfected MDCK cells. High molecular mass (200 kDa) appears to be a dimer of PSMA.

that PSMA is predominantly localized to the apical plasma membrane in both prostate tissue and polarized MDCK cells.

Tissue sections immunostained with antibody against PSCA revealed a different pattern of localization relative to PSMA. Matched tumor and morphologically normal tumor tissue sections revealed PSCA staining evident on both the apical and basolateral plasma membrane surfaces of prostatic epithelial cells (Fig. 6A). E-cadherin staining was observed at the basolateral plasma membrane in these tissues (data not shown). To investigate the expression of PSCA in MDCK cells, we generated a stable cell line expressing PSCA. Surface staining of these cells with antibody to PSCA and confocal analysis revealed PSCA to be distributed in a non-polarized manner at both the apical and basolateral plasma membrane surfaces (Fig. 6B and C).

Next, we examined polarized sorting of the secretory protein PSA in MDCK cells. PSA is secreted predominantly from the apical surface of secretory prostate epithelial cells into the lumen of the prostate gland. MDCK cells transiently transfected to express an epitope tagged version of PSA were grown on transwell filters. Following the formation of tight junctions, the PSA concentration in media from the apical and basolateral chambers was determined. The results

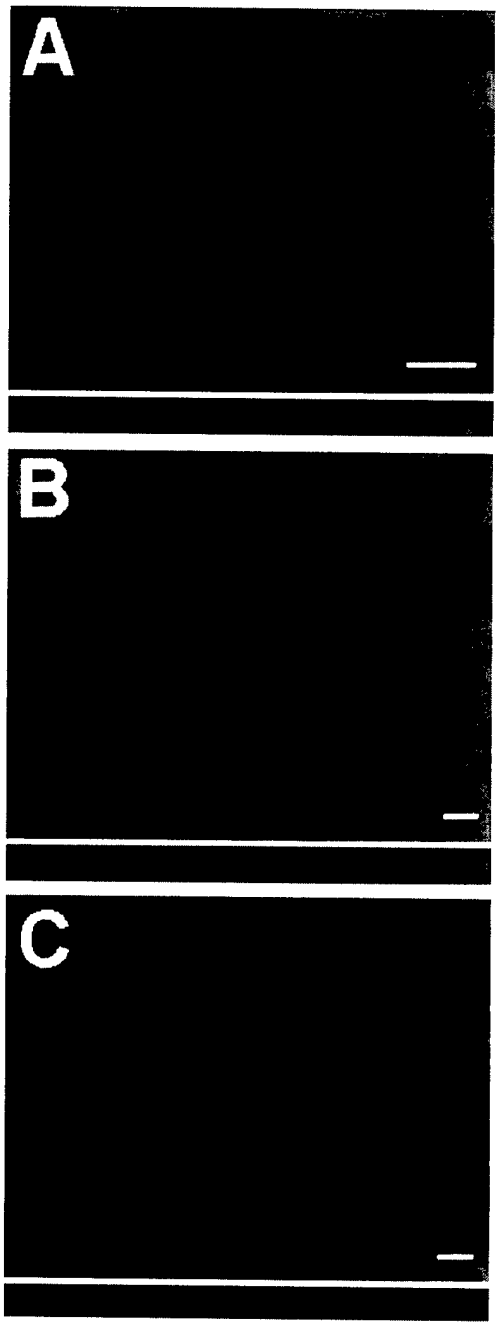


**Fig. 3.** Apical localization of PSMA in MDCK-PSMA cells. MDCK-PSMA cells were grown on glass coverslips and surface PSMA was labeled by incubating monolayers of cells on ice with mAb J591. Unbound J591 was removed and cells were fixed and incubated with CY3-conjugated secondary antibody. XY and XZ sections reveal PSMA to be localized to apical plasma membrane (**A**). Similar staining pattern observed for monolayers of MDCK-PSMA cells fixed in cold methanol and stained with mAb to GP-135 (an apical marker) and CY3-conjugated secondary antibody (**B**). Staining with mAb to β-catenin and CY3-conjugated secondary antibody reveals basolateral localization in XY and XZ sections (**C**). Bar, 10 μm.

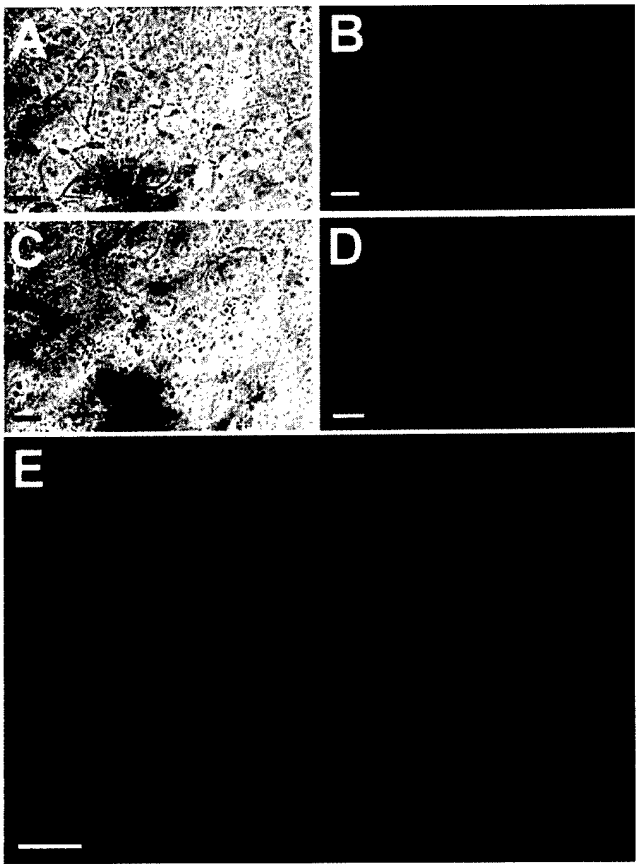


**Fig. 4.** Internalization of PSMA from the apical surface. Phase contrast images of confluent monolayers of MDCK-PSMA cells grown on glass coverslips are shown in **A** and **C**. In permeablized cells, mAb against E-cadherin and FITC-conjugated secondary antibody reveals a typical basolateral pattern (**B**). In non-permeablized cells, the presence of tight junctions restricts antibody from reaching E-cadherin at the basolateral surface, thus no staining is observed (**D**). Incubation of cells with mAb J591 at 37°C reveals a vesicular pattern, indicative of PSMA internalization from the apical surface. Bar, 10 μm.

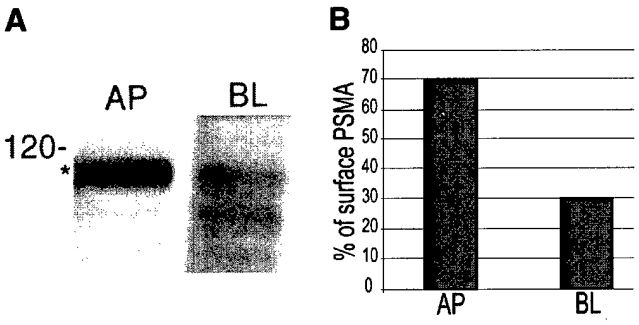


**Fig. 5.** Distribution of PSMA in MDCK-PSMA cells as revealed by a cell surface biotinylation assay. Monolayers of MDCK-PSMA cells were grown on transwell filters. Apical or basolateral surfaces were biotinylated and PSMA immunoprecipitated with mAb J591. SDS–PAGE and streptavidin-HRP analysis reveals surface PSMA to be localized primarily at the apical membrane (**A**). Quantification reveals 70% of cell surface PSMA to be apically localized (**B**).

presented in Figure 7A show that PSA is secreted primarily into the apical medium at an average of 2.3 times greater than into the basolateral medium.

To verify these results and demonstrate that these observations are not merely due to an artifact of transient transfection, stable MDCK cell lines expressing PSA were selected. Homogeneity of the clones was confirmed by immunofluorescence using an anti-*myc* antibody to epitope tagged PSA (data not shown). Two clones were plated on transwell filters and the formation of tight junctions was confirmed by measuring TER. Media from either chamber was analyzed for PSA concentration, and the results are shown in Figure 7B and C. Like in transiently transfected cells, two clones of MDCK-PSA cells showed 1.9–2.3 fold more secretion from the apical surface compared to the basolateral surface.

## DISCUSSION

In this study, we have used laser confocal microscopy to demonstrate that PSMA is localized to the apical surface of the prostatic epithelium, lining the lumenal interface of the gland. Through cell surface staining, internalization, and biotinylation assays, we have also shown that the majority of cell surface PSMA is similarly localized to the apical plasma membrane in MDCK-PSMA cells. Since the pattern of localization in MDCK cells is consistent with the pattern of expression seen within the prostatic epithelium, it is likely that the mechanisms responsible for apical targeting of this protein are conserved between these two cell types. Immunofluorescence performed on permeablized MDCK cells reveals a vesicular intracellular staining pattern in addition to apical plasma membrane staining, with no PSMA visible on the basolateral surface in regions of cell–cell contact. However, through a highly sensitive biotinylation assay, we were able to detect a low level of PSMA on the basolateral surface. Additional experiments are currently being conducted in our laboratory to evaluate the significance of this low steady state level of basolateral PSMA and elucidate the underlying mechanisms by which PSMA is targeted to the apical plasma membrane.

In order for epithelia to perform essential tasks of vectorial transport, epithelial cells must establish and maintain an asymmetric plasma membrane composition. Proteins destined for either the apical or basolateral surface must be selectively targeted to the appropriate plasma membrane domain. Proteins destined for either the apical or basolateral surface travel together through the cisternae of the Golgi apparatus to the *trans*-Golgi Network (TGN). In polarized epithelial cells, TGN is the site at which secretory and plasma

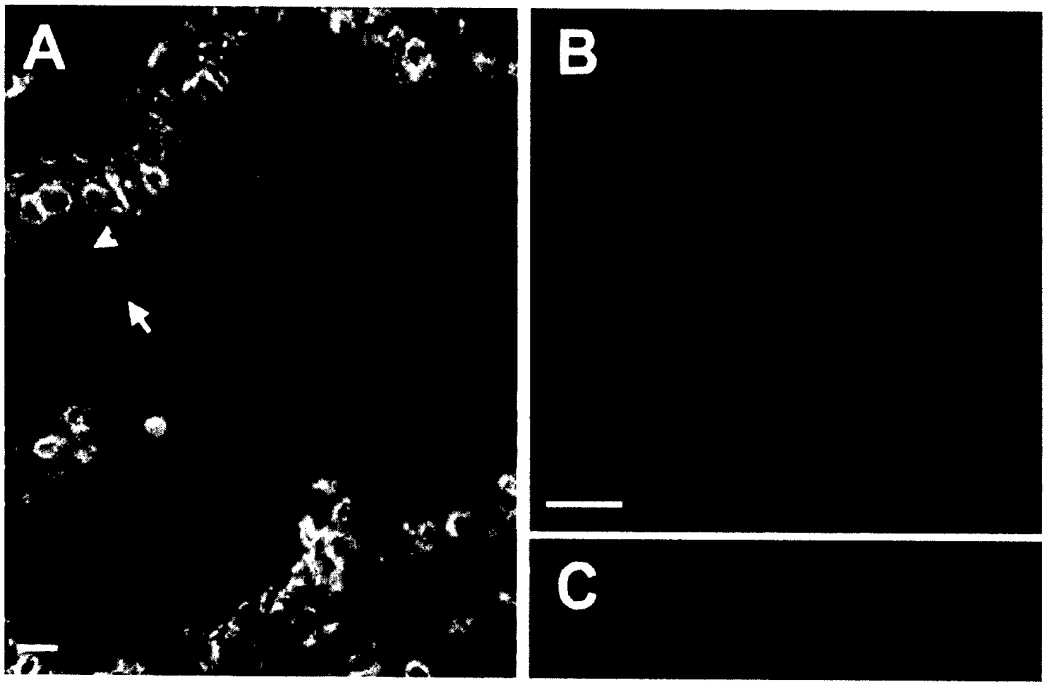


**Fig. 6.** Non-polarized distribution of PSCA in prostate tissue and MDCK cells. Normal tissue sections stained with propidium iodide to visualize nuclei (red), mAb IG8, and FITC-conjugated secondary antibody (green) show PSCA to be present on both apical and basolateral plasma membrane surfaces. PSCA on the apical plasma membrane is indicated with an arrow, basolateral with an arrowhead (**A**). IG8 was used to perform cell surface staining on confluent monolayers of MDCK-PSCA cells grown on transwell filters. Confocal microscopy reveals PSCA to be localized in a non-polarized fashion on apical and basolateral plasma membrane surfaces (**B** and **C**). Bar, 10 μm.

membrane proteins are sorted to the apical and basolateral plasma membrane domains [39]. In the TGN, sorting mechanisms interpret signals encoded within the proteins and properly direct them into discrete vesicles destined for the appropriate membrane domain [40]. Different cell types utilize different repertoires of sorting mechanisms, emphasizing the importance of choosing an appropriate model system to study protein sorting in polarized epithelia [40].

The signals responsible for the targeting of proteins to basolateral plasma membrane are thought to reside exclusively within the cytoplasmic domain. Among

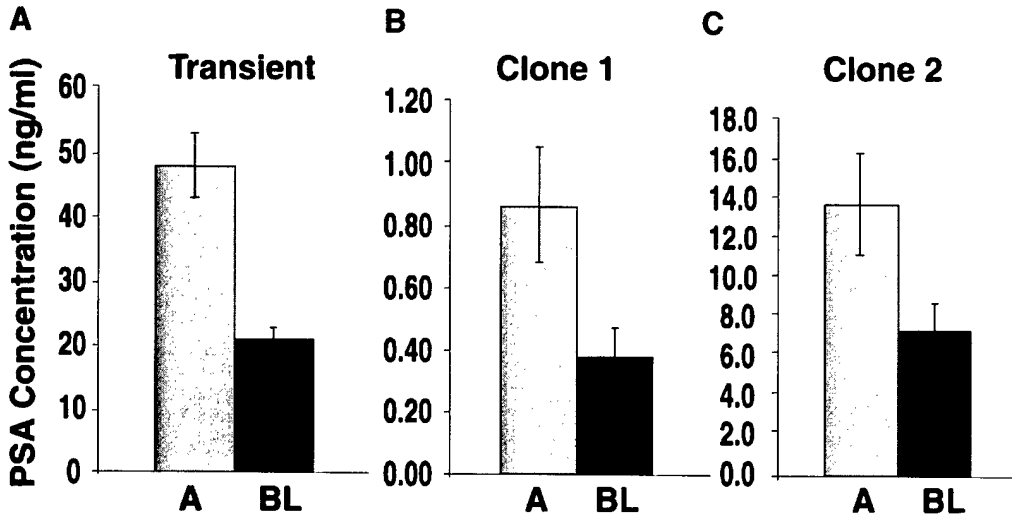


**Fig. 7.** Apical secretion of PSA in transfected MDCK cells. PSA is secreted predominantly form the apical surface either in transiently transfected cells (**A**) or in stable cell clones (**B** and **C**) expressing PSA. Results represent the average of 4 – 5 independent determinations in each case. Error bars represent standard error.

the best-characterized signals are the tyrosine- and dileucine-based motifs. These signals were originally identified through their ability to mediate internalization of proteins via clathrin coated pits, and have since been shown to interact with adaptor protein complexes. In contrast, the signals responsible for apical sorting have remained much more enigmatic. Reports have shown that a variety of molecular determinants may be involved in apical targeting, including GPI anchors, lipid rafts, N-glycosylation, O-glycosylation, and amino acid motifs encoded within the cytoplasmic or transmembrane domains. The characterization of MDCK cells as a model in which to study the sorting of PSMA should enable us to further define the mechanisms of apical targeting within epithelia. While the cytoplasmic tail of PSMA contains a dileucine-like motif the majority of cell-surface PSMA is localized to the apical plasma membrane, indicating that the transmembrane or extracellular domain of PSMA contain a dominant apical targeting signal. This possibility is currently being explored in our laboratory.

While PSMA was distinctly localized to the apical plasma membrane in most of the prostate tumor tissues examined in this study, we observed localization to the basolateral surface in tissue sections derived from two patients (Fig. 1C). Although the fluorescence intensity of the basolaterally expressed PSMA was lower than the apically localized PSMA in this sample, this result indicates that PSMA can also be targeted to the basolateral plasma membrane. The presence of PSMA on the basolateral plasma membrane may have important clinical significance. PSMA on the basolateral surface would be in closer proximity to the vasculature and thus these patients may exhibit improved response to immunotherapy. Further study will be required to evaluate this hypothesis.

The normal polarized localization of E-cadherin in the same glands that showed PSMA localized on the basolateral surface indicates that the change in polarity of PSMA is specific, and not due to a general loss of polarity or tight junction integrity. Since E-cadherin is associated with the actin cytoskeleton through its interaction with catenins [41], it is possible that the association with the actin cytoskeleton retained this protein at the basolateral plasma membrane in spite of aberrant sorting mechanisms in these tumor cells. According to this possibility, the apical localization of PSMA may be affected while the basolateral localization of E-cadherin should be maintained. Alternatively, PSMA in this tumor tissue may have a different glycosylation pattern or a mutation in the apical targeting signal, which would lead to the basolateral localization of PSMA. It is also possible that abundant levels of PSMA expressed in these tumor cells might have resulted in the saturation of the apical targeting machinery resulting in the localization of apical proteins in the basolateral plasma membrane. While additional tissue samples must be examined to further determine whether such basolateral localization of PSMA is a general phenomenon in the progression of prostate cancer, the availability of MDCK cells as a model to study polarized sorting should permit investigations into these possibilities.

We have also investigated the localization of a second membrane associated, prostate restricted antigen, PSCA. Confocal microscopy reveals that PSCA is distributed in a non-polarized manner within the prostatic epithelium. In MDCK cells, PSCA is similarly distributed to both apical and basolateral plasma membrane surfaces. The similar patterns of localization provide further evidence that MDCK cells are a useful model in which to study polarized sorting in the prostatic epithelium.

The non-polarized plasma membrane distribution of PSCA was an unexpected result for a GPI-anchored protein. In MDCK and many other epithelial cell types, the presence of such GPI-modifications has been shown to specifically target proteins to the apical plasma membrane. This targeting presumably occurs via association with membrane microdomains known as lipid rafts [42]. Exactly why PSCA is distributed in such a non-polarized manner has yet to be determined. Elucidating the targeting information encoded within PSCA and determining the ability of this GPI-anchored protein to associate with lipid rafts will be the subject of future research.

In this report, we have also studied the polarized secretion of the prostate restricted protease, PSA. Within the prostate, PSA is secreted apically from the epithelium into the lumen of the gland [23]. Low levels of PSA are also found within the blood, suggesting that a fraction of PSA may be secreted from the basolateral surface, where it can gain access to the vasculature [27]. At the levels of PSA expression observed in this study, PSA is secreted from the apical surface at levels 2–2.3 times greater than from the basolateral plasma membrane. These data are again consistent with in vivo observations suggesting that the mechanisms of apical secretion of PSA are conserved in prostate tissue and in MDCK cells.

Non-polarized secretion of proteases, such as PSA, may contribute to metastasis by degrading extracellular matrix proteins. In fact, Webber et al. demonstrated that blocking PSA activity with antibodies reduced the invasiveness of LNCaP cells, in vitro [25]. In addition, aberrantly polarized secretion of proteases may also promote cell growth by activating mitogens associated with the extracellular matrix. In this regard, PSA has been shown to activate latent growth factors, such as transforming growth factor β and insulin-like growth

factor I [43,44]. Although increased serum levels of PSA have long been known to be associated with prostate cancer, the mechanism by which this occurs is not well understood. This phenomenon could be due to saturation of the apical secretory pathway, loss epithelial polarity, a specific defect in the targeting pathway, or to the direct secretion of PSA into the serum by either invading or circulating metastatic cells. The use of MDCK cells as a model system may provide insight into the mechanism of increased PSA serum levels and help to further define role(s) for PSA in prostate cancer.

Studying the patterns and mechanisms of epithelial polarity can provide invaluable insight into normal physiology as well as the pathophysiology of disease. Understanding the extracellular environment (apical or basal) to which a protein is exposed can provide information regarding the normal physiological role of that protein. In addition, loss of polarity may provide the molecular basis for a number of pathological conditions. One of the hallmarks of cancer is the loss of normal epithelial phenotype. Very often, the progression of tumors to more aggressive phenotypes is closely associated with a concomitant loss of tight junctions and epithelial polarity [45]. Aberrant targeting of enzymes and receptors or the loss of polarity may have significant impact on disease and tumor progression. Expression of receptors within an inappropriate extracellular milieu may lead to aberrant stimulation of signaling events that could potentially provide a mitogenic stimulus. Loss of tight junctions in cancer cells may lead to aberrant surface expression of domain specific proteins, as evidenced by the non-polarized distribution of E-cadherin in transformed MDCK cells lacking tight junctions [36]. MDCK cells offer a useful system in which to study the role of tight junctions in polarized sorting of PSMA and PSA, as the permeability of these junctions can be affected by altering the extracellular calcium levels or by other mechanisms. Ultimately these studies should provide valuable insight into the function of these proteins in normal tissue and in disease.

## ACKNOWLEDGMENTS

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# Prostate-specific Membrane Antigen Association with Filamin A Modulates Its Internalization and NAALADase Activity[1]

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## ABSTRACT

**Prostate-specific membrane antigen (PMSA) is an integral membrane protein highly expressed by prostate cancer cells. We reported previously that PSMA undergoes internalization via clathrin-coated pits (Liu *et al.*, Cancer Res., *58:* 4055–4060, 1998). In this study we demonstrate that filamin A, an actin cross-linking protein, associates with the cytoplasmic tail of PSMA and that this association of PSMA with filamin is involved in its localization to the recycling endosomal compartment. By ectopically expressing PSMA in filamin-negative and -positive cell lines, we additionally show that filamin binding to PSMA reduces the internalization rate of PSMA and its *N*-acelylated-α linked-acidic dipeptidase activity. These results suggest that filamin might be an important regulator of PSMA function.**

## INTRODUCTION

PSMA[4] is a type-II integral membrane protein of $M_r$ 100,000 with a short 19 amino acid cytoplasmic tail, predominantly localized to the epithelial cells of the prostate gland (1, 2). PSMA has been shown to have two enzymatic activities; folate hydrolase (3) and NAALADase (4). In normal prostate epithelial cells, expression of PSMA is very low, and the level increases several fold in high-grade prostate cancers, metastatic diseases, and hormone-refractory prostate carcinoma (5). mAbs raised against the extracellular domain of PSMA have been conjugated with either radioactive ligands or cytotoxins for use as immunotoxins for the specific targeting of prostate cancer cells (6). The importance of PSMA was additionally illustrated by the finding that it is also expressed in endothelial cells of the neovasculature but is absent in normal endothelial cells (7).

PSMA is constitutively internalized via clathrin-coated pits in LNCaP cells (8). In addition, antibody specific for PSMA extracellular domain increased the rate of internalization of PSMA (8). Deletion of the cytoplasmic tail of PSMA resulted in the loss of internalization of PSMA, indicating that the cytoplasmic tail is crucial for its internalization.[5] To additionally understand the mechanism of PSMA internalization we used the cytoplasmic tail of PSMA as bait in a yeast two-hybrid screening approach to identify PSMA interacting proteins. In this study we report that the cytoplasmic tail of PSMA associates with filamin A, an actin cross-linking phosphoprotein and that this association modulates the internalization rate of PSMA and its localization to the REC. Our studies also suggest that PSMA-filamin association is involved in the regulation of NAALADase activity of PSMA.

## MATERIALS AND METHODS

**Antibodies and Reagents.** Mouse mAb, J591, against the extracellular domain of PSMA has been described (7). Mouse mAb against filamin A was from Chemicon International Inc. (Temecula, CA). FITC- and Texas Red-labeled, affinity-purified secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA), and horseradish peroxidase-conjugated antimouse antibody and streptavidin were from Transduction Laboratories. PC3 cells stably expressing PSMA have been described earlier (9) and were kindly provided by Dr. Warren Heston (Cleveland Clinic Foundation, Cleveland, OH). M2 and A7 melanoma cell lines were generously provided by Dr. Thomas Stossel (Harvard Medical School, Boston, MA).

**Cell Culture and Transfections.** PC3-PSMA cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, MEM nonessential amino acid solution (Invitrogen, Grand Island, NY), and penicillin/streptomycin. A7 and M2 cells were grown in MEM containing 8% newborn FCS, 2% fetal bovine serum, MEM nonessential amino acid solution, and penicillin/streptomycin. The calcium phosphate method was used for all of the transfections (10).

**Yeast Two-Hybrid Analysis.** Two complementary oligonucleotides, representing the 19 amino acid cytoplasmic tail of PSMA, were synthesized, annealed, and cloned into the bait vector pGBKT7. This bait was used to screen a human prostate-specific cDNA library using a yeast strain AH109. Library screening and β-galactosidase assays were performed as per the guidelines of the Matchmaker Two-Hybrid System 3 of Clontech (Palo Alto, CA).

**GST Pull-Down and Immunoprecipitation.** A 520-bp DNA fragment corresponding to the $NH_2$-terminal 1–173 amino acids of PSMA was cloned in fusion with GST in the pGEX-4X vector. The GST-PSMA fusion protein overexpressed in *Escherichia coli* was purified to homogeneity according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ). DNA representing the 23rd-24th repeats (2466–2647 amino acids) of filamin A was cloned in pET 28-b, a T7-based bacterial expression vector. This construct was used to generate $^{35}S$-labeled filamin A in a coupled *in vitro* transcription and translation system obtained from Promega (Madison, WI). The GST pull-down assay was carried out in a 50 μl reaction volume [binding buffer: 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 12.5 mM $MgCl_2$, and 10% glycerol] containing 500 ng of affinity purified GST-PSMA, and 5 μl of *in vitro* transcription and translation reaction containing radioactive filamin A. The binding reaction was carried out at 4°C for 2 h, and the bound filamin was pulled down using GST beads. Purified GST protein incubated with radioactive filamin A was used as a control in the assay. Bound proteins were analyzed by 15% SDS-PAGE followed by autoradiography.

For immunoprecipitation, PC3-PSMA were rinsed with ice-cold PBS and lysed in 1 ml of lysis buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton-X-100, 40 mM *N*-octylglucoside, 0.2 mM sodium vanadate, 1 mM EDTA, 1 mM EGTA, 50 μg/ml DNase, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml each of antipapain, leupeptin, and pepstatin] on ice for 30 min. The lysates were subjected to immunoprecipitation using and mAb J591 and immunoblotted with antifilamin antibody.

**Immunofluorescence and Confocal Microscopy.** Immunofluorescence and confocal microscopy were performed as described earlier (10). Cells were grown to 70% confluency on coverslips, washed twice using PBS, and fixed with methanol. After blocking with BSA, cells were incubated with antifilamin mAbs for 1 h at room temperature, washed, and counterstained with Cy3-conjugated rabbit antimouse secondary antibody for visualizing filamin. FITC-conjugated J591 anti-PSMA antibody was used for the PSMA staining. To detect FITC- and Cy3-labeled antigens, samples were excited at 488 nm and

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[2] N. H. B. is a paid consultant of BZL, Inc. (Framingham, MA), and has developed mAb J591 used in this study, which was patented by the Cornell Research Foundation and licensed to BZL.

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[4] The abbreviations used are: PSMA, prostate-specific membrane antigen; REC, recycling endosomal compartment; NAAG, *N*-acetyl-aspartyl glutamate; NAALADase, *N*-acelylated-α linked-acidic dipeptidase; mAb, monoclonal antibody; GST, glutathione *S*-transferase.

[5] S. Rajasekaran, G. Anilkumar, and A. K. Rajasekaran, unpublished observations.

550 nm, and the light emitted between 520–540 nm and 560–610 nm, respectively, was recorded. Colocalization of filamin A and PSMA was carried out using a Fluoview laser scanning confocal microscope (Olympus America Inc., Melville, NY). Images were generated using Fluoview software (version 2.1.39).

**Internalization of PSMA.** Internalization of PSMA was determined by a cell surface biotinylation assay as described previously (8).

**NAALADase Assay.** Radioenzymatic assay using [$^3$H]NAAG (NEN, Boston, MA) was performed as described previously (11). A7 and M2 melanoma cells stably expressing PSMA were incubated in 500 $\mu$l of Krebs Ringer bicarbonate medium containing 1 $\mu$Ci/ml [$^3$H]NAAG at 37°C for 1 h. After the incubation, the cells were placed on ice and washed three times with ice-cold Krebs Ringer medium. The cells were lysed using 1 M NaOH, and the radioactivity was determined using a scintillation counter (Beckman LK 6500). The counts were normalized with the total amount of PSMA expressed on the cell surface determined by cell surface biotinylation.

## RESULTS

**Identification of PSMA Binding Partners Using Yeast Two-Hybrid Analysis.** The 19 amino acid cytoplasmic $NH_2$ terminus of PSMA was used as a bait to screen a human prostate cDNA library in yeast strain AH 109. Several rounds of library screening ($1.4 \times 10^6$ clones) identified a number of interacting clones that grew on selective media indicating the presence of interactors for the cytoplasmic domain of PSMA. Among all of the positive clones sequenced, 50% represented two distinct COOH-terminal regions, corresponding to the 22–24$^{th}$ and 23–24$^{th}$ repeats of filamin A. Filamin has three isoforms: filamin A (also called ABP 280, filamin $\alpha$, Filamin), filamin B (also called ABP278, filamin$\beta$), and filamin C (also called ABPL, filamin $\gamma$; Ref. 12). We also obtained partial cDNA clones identical to the COOH-terminal region of filamin B and filamin C (Fig. 1*B*). The amino acid sequence alignment of the regions of the three isoforms of filamin that interacted with the cytoplasmic tail of PSMA encompassed their 22–24$^{th}$ repeat as shown in Fig. 1*A*. There was 70% identity at the amino acid level among the different filamin isoforms in this region.

**PSMA Binds to Filamin *in Vitro* and *in Vivo*.** An *in vitro* GST pull-down assay was carried out to determine whether PSMA interacted directly with filamin A. As shown in Fig. 1*C* the interaction between PSMA and filamin was readily detected. Coimmunoprecipitation analysis was performed to determine whether filamin and PSMA associate *in vivo*. PC3-PSMA cells were lysed in an immunoprecipitation buffer, and PSMA was immunoprecipitated by mAb J591. Coimmunoprecipitating filamin was detected by antifilamin antibody. In agreement with the two-hybrid and bacterial fusion protein analyses, filamin was coimmunoprecipitated *in vivo* from prostate epithelial cells (Fig. 1*D*).

**Colocalization of PSMA and Filamin in PC3-PSMA Cells.** Confocal microscopy revealed that PSMA colocalized with filamin at the plasma membrane region (Fig. 2, *A–C*) indicating that PSMA and filamin associate at the plasma membrane. In addition to its plasma membrane localization, PSMA also exhibited a distinct spot-like staining at the perinuclear region (Fig. 2*B*, *arrowhead*), a staining pattern similar to that of the recycling endosome (13). Therefore, to examine whether PSMA is localized to the recycling endosome, we tested whether the PSMA codistributes with a recycling endosomal marker (internalized transferrin). The internalized transferrin clearly colocalized with PSMA in the perinuclear region (Fig. 2, *D–F*), indicating that PSMA is localized to the REC in PC3-PSMA cells.

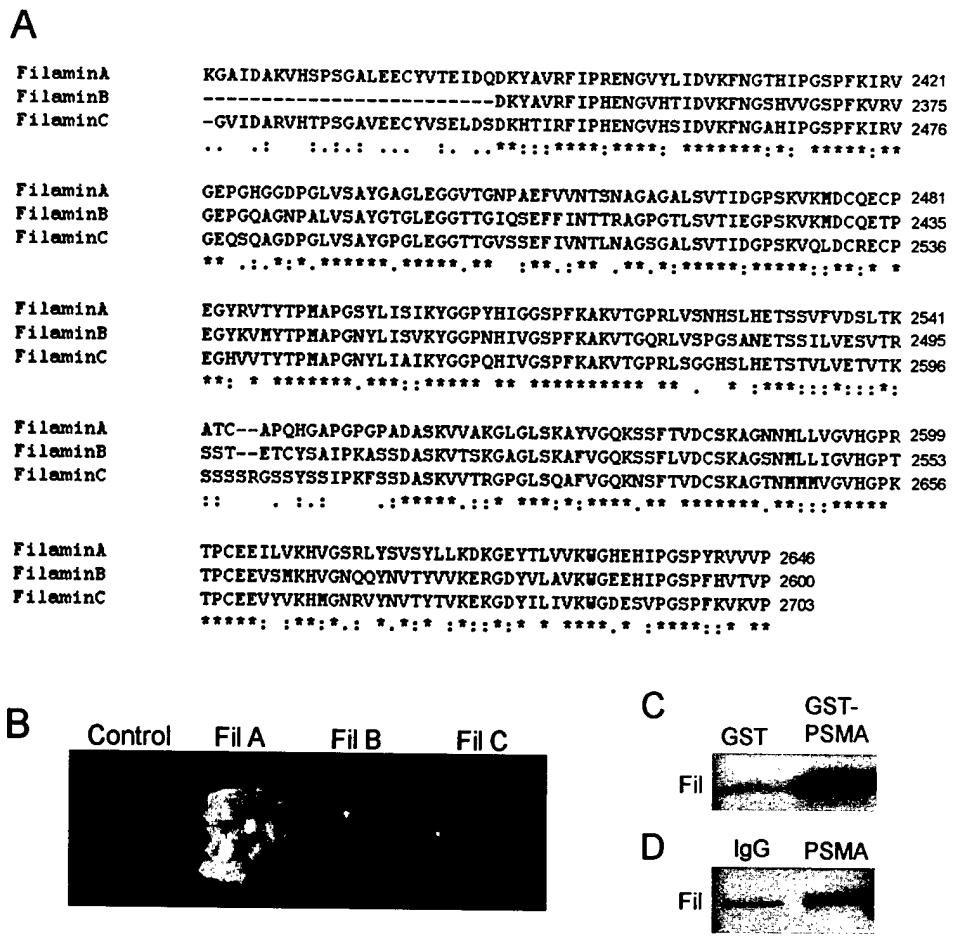


Fig. 1. Association of PSMA with filamin *in vitro* and *in vivo*. *A*, amino acid alignment of regions of filamin A, B, and C that interact with the cytoplasmic tail of PSMA in the yeast two-hybrid screening. * represents identical amino acids in all three of the filamins; *colons* represent conserved substitutions. *B*, growth of yeast expressing the bait alone (*control*) or the COOH-terminal region of filamin A, B, or C cDNA clones on medium lacking tryptophan, leucine, histidine, and adenine. *C*, filamin A and PSMA interact directly *in vitro*. An $NH_2$-terminal region of PSMA was expressed in *E. coli* as a GST fusion product (*GST-PSMA*) and was used to pull-down the COOH-terminal region of filamin A. Filamin A was generated as described in "Materials and Methods." GST protein alone was used as a control (*GST*). *D*, association of PSMA and filamin A *in vivo*. Protein lysate was prepared from PC3 cells expressing PSMA, and total PSMA was immunoprecipitated with mAb J591 antibody or control IgG and immunoblotted for filamin.

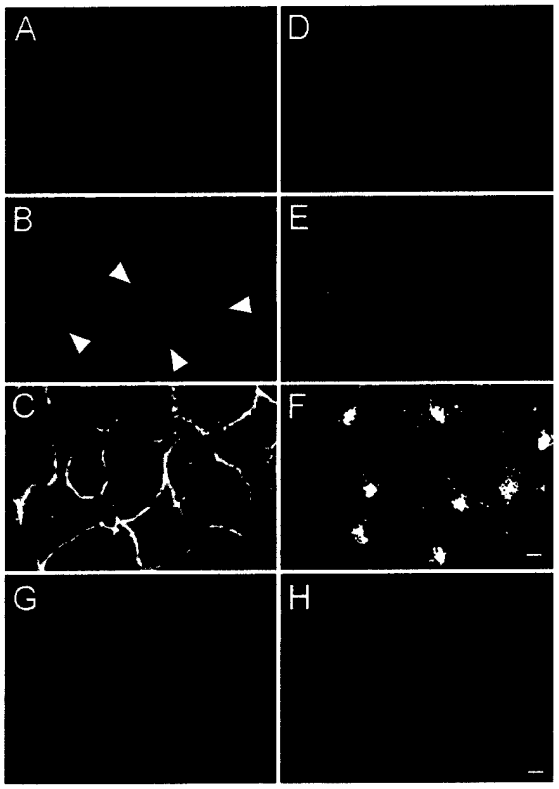


Fig. 2. Colocalization of PSMA with filamin A and localization of internalized PSMA to the REC. Confocal microscope optical sections of PC3-PSMA cells stained for filamin A (*A*) and PSMA (*B*). The merged image is shown in *C*. *Arrowheads* indicate the REC. FITC-labeled transferrin uptake (*D*), PSMA internalization as revealed by the uptake of mAb J591 (*E*), and (*F*) the merged image of *D* and *E*. Note the accumulation of transferrin and PSMA in the REC. Localization of internalized PSMA in filamin A-negative M2 (*G*) and filamin A-positive A7 cells (*H*). *Bar*, 10 μm.

**Localization of PSMA in Filamin-negative and -positive Cell Lines.** Next, we tested whether PSMA-filamin association is involved in the localization of PSMA to the REC. Because PC3 cells contain filamin, we used M2 cells, a cell line derived from human malignant melanoma, which is negative for filamin expression, and A7 cells, a subline generated by stable transfection of M2 with filamin cDNA. These cells do not express endogenous PSMA, and, therefore, PSMA was ectopically expressed in these cell lines. Strikingly, in filamin-negative M2 cells, PSMA showed vesicular staining throughout the cytoplasm (Fig. 2*G*), whereas in A7 cells, a distinct spot-like staining (Fig. 2*H*), as seen in PC3-PSMA cells (compare Fig. 2, *B* and *H*) was observed. These results indicate that filamin is involved in the localization of PSMA to the REC.

**Decreased Internalization of PSMA in Filamin-positive Cells.** Localization of PSMA to the REC in filamin-positive A7 cells indicated that PSMA filamin association might affect internalization of PSMA. Therefore, we tested the rate of internalization of PSMA in M2 and A7 cells using a cell surface biotinylation assay. M2 cells showed ~2-fold increase in the internalization of PSMA (Fig. 3, *A* and *B*). Strikingly, at 60 min, 100% of the total surface PSMA was internalized in M2 cells. In A7 cells, at 60 min there was a slight decrease in the internalized PSMA as compared with the 30-min time point, which is possibly because of degradation. These results indicate that filamin might be involved in the modulation of internalization rate of PSMA.

**Increased NAALADase Activity of PSMA in Filamin-deficient Cells.** We then tested whether PSMA association with filamin is involved in the regulation of its NAALADase activity using PSMA expressing M2 and A7 cells. NAALADase activity was determined using [$^3$H]NAAG as the substrate. The activity was normalized to the amount of cell surface PSMA determined by cell surface biotinylation assay. The NAALADase activity was 1.5-fold higher in M2 cells compared with A7 cells (Fig. 4) indicating that the filamin association decreases the enzymatic activity of PSMA.

## DISCUSSION

In this study, we show that the first 19 $NH_2$-terminal amino acids of PSMA associate with the 23$^{rd}$-24$^{th}$ repeat of filamin A. This interaction between PSMA and filamin A found in the yeast two-hybrid analysis was confirmed using *in vitro* pull-down assays using GST-PSMA, and *in vivo* coimmunoprecipitation and colocalization experiments. We confirmed that PSMA is localized to the REC in filamin-positive cells. We also provided biochemical evidence that in the absence of filamin association, PSMA is internalized faster and exhibits increased NAALADase activity.

Filamin A is a dimeric actin cross-linking phosphoprotein located in the cortical cytoplasm adjacent to the plasma membrane. Parallel filamin dimers cross-link actin and facilitate the orthogonal branching of actin filaments, and serve as a docking site for various cell surface receptors and certain intracellular proteins involved in signal trans-

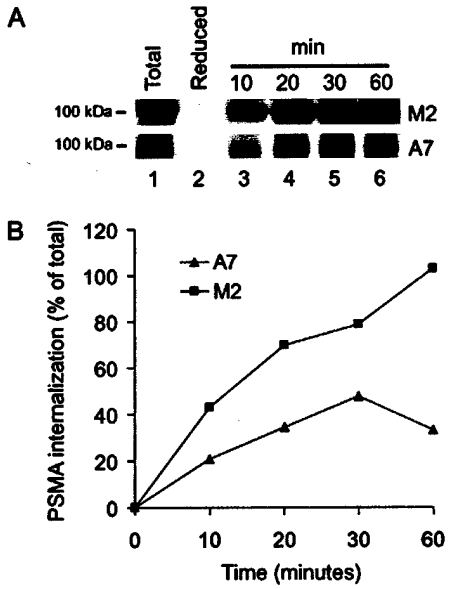


Fig. 3. Increased internalization of PSMA in a filamin-negative cell line. (*A*) The cell surface was biotinylated as described in Material and Methods. PSMA was immunoprecipitated at different time points, run on a 10% SDS-PAGE and internalized PSMA was detected with HRP-streptavidin. (*B*) Densitometric quantitation showed an approximately 50% reduction in the rate of internalization of PSMA in filamin positive cells (A7) compared with filamin negative cells (M2).

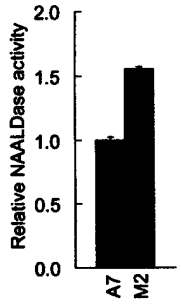


Fig. 4. Increased NAALADase activity in filamin-negative cells. A7 and M2 cells stably expressing PSMA were used for NAALADase assay. Note the 1.5-fold increase of NAALADase activity of PSMA in filamin-negative cells (*M2*); *bars*, ±SD.

duction (12). In this study, we have shown that PSMA association with filamin is necessary for its localization to the REC. In filamin-negative M2 cells, the internalized PSMA accumulated in vesicles, which were highly diffused throughout the cytoplasm. This diffused staining pattern changed into a bright spot like perinuclear staining (REC) in cells transfected with filamin A cDNA (A7 cells). REC is a collection of 50–70 nm diameter tubules containing recycling receptors and some ligands that are known to undergo recycling, and is generally found to localize near the microtubule-organizing center (14). Our results are consistent with a view that PSMA filamin association is involved in the transport of PSMA from the cell surface to the REC. However, we do not rule out the possibility that filamin might also be involved in the organization and maintenance of REC in mammalian cells. Additional experiments are necessary to unravel the role of filamin in targeting PSMA to REC and its possible involvement in the regulation of REC structure.

LNCaP cells, which express endogenous PSMA and filamin, did not show localization of PSMA to the REC, and PSMA did not coimmunoprecipitate with filamin.[6] It is possible that PSMA association with filamin is modulated by intracellular signaling mechanisms. The PSMA cytoplasmic tail has three putative phosphorylation sites. It is known that filamin A undergoes phosphorylation *in vivo*, and this regulates its interaction with the actin cytoskeleton (15). Whether the phosphorylation status of filamin or PSMA is different in LNCaP compared with PC3 cells and whether this modulates PSMA localization to the REC remains to be studied.

PSMA association with filamin A decreased its rate of internalization by ~50%. This is in agreement with an earlier observation that endocytosis of furin, an endoprotease, was reduced 42% upon binding with filamin A (16). This may occur by the stabilization of these molecules on the membrane by linking to the actin cytoskeleton via filamin A. Dissociation from filamin might facilitate the binding of adaptor proteins required for endocytosis. Liu *et al.* (16) suggested the existence of a competition between filamin A and adaptors binding to the same site of furin, thereby regulating the rate of internalization of furin. Our observations that mutation of specific cytoplasmic tail amino acid residues of PSMA resulted in the loss of its internalization[5] and that these mutants interacted more strongly with filamin A[6] support the above possibility.

In our study, we have shown that filamin binding reduced the NAALADase activity of PSMA by 64%. It is possible that the interaction between filamin A and the cytoplasmic tail of PSMA changes the conformation of the extracellular domain of PSMA, resulting in reduced substrate (NAAG) binding. The observation that the cytoplasmic tail of a protein regulates the conformation of its extracellular domain has been reported previously for calreticulin, a $M_r$ 60,000 intracellular calcium-binding protein. The cytoplasmic tail of calreticulin interacts with a highly conserved GFFKR motif present in all of the $\alpha$-subunits of integrin cytoplasmic domains, and promotes integrin to maintain a high affinity state for ligand binding (17).

The significance of the internalization of PSMA in prostate epithelial cells is not known. Antibody-induced endocytosis, high similarity at amino acid level between transferrin receptor and PSMA, and the presence of a domain similar to the transferrin receptor dimerization domain at the extracellular region of PSMA strongly suggest that PSMA could be a membrane receptor for an unknown ligand. On binding to ligand, the PSMA-ligand complex could undergo internalization. At present, there is no clue whatsoever about the nature of the ligand. If PSMA transduces a cell growth advantage signal, then its proper down-regulation by endocytosis would be important to prevent transformation of normal cells into cancerous cells. This is observed in the case of epidermal growth factor receptor. An epidermal growth factor receptor mutant unable to undergo endocytosis has been identified in human cancers (18). Endocytosis of PSMA might also be important for its role in the metastasis of prostate cancer. In this case, an increased internalization of PSMA could promote the invasive property of prostate cancer cells. It is reported that cytoplasmic tail-dependent internalization of membrane-type I matrix metalloproteinase was important for its invasion-promoting activity (19). We are currently carrying out experiments to delineate the potential importance of endocytosis of PSMA in prostate cancer.

[6] G. Anilkumar and A. K. Rajasekaran, unpublished observations.

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# A Novel Cytoplasmic Tail MXXXL Motif Mediates the Internalization of Prostate-specific Membrane Antigen

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**Prostate-specific membrane antigen (PSMA) is a transmembrane protein expressed at high levels in prostate cancer and in tumor-associated neovasculature. In this study, we report that PSMA is internalized via a clathrin-dependent endocytic mechanism and that internalization of PSMA is mediated by the five N-terminal amino acids (MWNLL) present in its cytoplasmic tail. Deletion of the cytoplasmic tail abolished PSMA internalization. Mutagenesis of N-terminal amino acid residues at position 2, 3, or 4 to alanine did not affect internalization of PSMA, whereas mutation of amino acid residues 1 or 5 to alanine strongly inhibited internalization. Using a chimeric protein composed of Tac antigen, the α-chain of interleukin 2-receptor, fused to the first five amino acids of PSMA (Tac-MWNLL), we found that this sequence is sufficient for PSMA internalization. In addition, inclusion of additional alanines into the MWNLL sequence either in the Tac chimera or the full-length PSMA strongly inhibited internalization. From these results, we suggest that a novel MXXXL motif in the cytoplasmic tail mediates PSMA internalization. We also show that dominant negative μ2 of the adaptor protein (AP)-2 complex strongly inhibits the internalization of PSMA, indicating that AP-2 is involved in the internalization of PSMA mediated by the MXXXL motif.**

## INTRODUCTION

Prostate-specific membrane antigen (PSMA) was originally identified by the monoclonal antibody (mAb) 7E11-C5 raised against the human prostate cancer cell line LNCaP (Horoszewicz *et al.*, 1987). Subsequently, the PSMA gene was cloned (Israeli *et al.*, 1993) and mapped to chromosome 11q (Rinker-Schaeffer *et al.*, 1995). PSMA is a type II membrane protein with a short cytoplasmic N-terminal region (19 amino acids), a transmembrane domain (24 amino acids), and a large extracellular C-terminal portion (707 amino acids) (Israeli *et al.*, 1993) with several potential N-glycosylation sites. Recently, it has been shown that PSMA is homologous to glutamate carboxypeptidase II (85% at nucleic acid level) isolated from rat brain (Coyle, 1997) and has folate hydrolase activity (Pinto *et al.*, 1996; Halsted *et al.*, 1998), and N-acetylated α-linked acidic dipeptidase (NAALDase) activity (Carter *et al.*, 1996, 1998). The extracellular domain of PSMA shows homology (26% identity at the amino acid level) to the transferrin receptor I (Israeli *et al.*, 1993) and to a recently cloned transferrin receptor II (Kawabata *et al.*, 1999). The functional significance of homology between PSMA and transferrin receptor is not known.

PSMA has been the subject of increasing interest in cancer research due to its potential as a diagnostic and therapeutic target for human prostate cancer (Chang *et al.*, 1999a). PSMA is abundantly expressed in prostate cancer cells. Its expression is further increased in higher-grade cancers, metastatic disease, and hormone-refractory prostate carcinoma (Wright *et al.*, 1996; Silver *et al.*, 1997). In addition, PSMA has become the focus of even more intense interest due to the recent findings that it is selectively expressed in the neovasculature of nearly all types of solid tumors, but not in the vasculature of normal tissue (Liu *et al.*, 1997; Silver *et al.*, 1997; Chang *et al.*, 1999b,c). The function of PSMA with respect to vascular endothelial cell biology and the direct correlation between its expression and increasing tumor aggressiveness in prostate cancer remain intriguing and unclear. Although a significant amount of research is being carried out using antibodies against PSMA for immunotherapy of prostate cancer (McDevitt *et al.*, 2001; Smith-Jones *et al.*, 2003), very little is known about the mechanism of internalization of this protein.

In general, the endocytic pathway includes internalization of the receptor-ligand complex via clathrin-coated pits and accumulation in the endosomes. The receptor–ligand complex then dissociates in the endosomes and the dissociated molecules are either recycled back to the cell surface or targeted to lysosomes for degradation (Pastan and Willing-

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Abbreviations used: NAALDase, N-acetylated α-linked acidic dipeptidase; PSMA, prostate-specific membrane antigen.

ham, 1981; Mellman, 1996). Targeting of most receptors to coated pits and their traffic through endocytic compartments are generally mediated by endocytic signals located in the cytoplasmic domain of proteins (reviewed in Trowbridge *et al.*, 1993; Kirchhausen *et al.*, 1997; Mukherjee *et al.*, 1997; Bonifacino and Traub, 2003). These signals fall into two major categories such as tyrosine-based and di-leucine based signals.

The tyrosine-based signals are represented by NPXY and YXXΦ consensus motifs (Bonifacino and Traub, 2003) with the Y residue being critical for their function (Lazarovits and Roth, 1988). NPXY signals mediate internalization of several type-1 membrane proteins such as LDL receptor, epidermal growth factor receptor, insulin receptor, and others (Bonifacino and Traub, 2003). The YXXΦ (Φ, bulky hydrophobic side chain) signals mediate internalization and lysosomal targeting of several type I and type II membrane proteins such as transferrin receptor, mannose-6-phosphate receptor, asialoglycoprotein receptor, polymeric immunoglobulin receptor, and others (reviewed in Trowbridge *et al.*, 1993; Marks *et al.*, 1997; Bonifacino and Dell'Angelica, 1999; Bonifacino and Traub, 2003).

The di-leucine based signals require two consecutive leucines or a leucine-isoleucine pair for their function (Letourneur and Klausner, 1992; Sandoval and Bakke, 1994). Recent studies have identified two distinct classes of di-leucine based signals represented by [DE]XXXL[LI] and DXXLL consensus sequences. Both these signals are involved in internalization and lysosomal targeting of several membrane proteins. Proteins such as CD3-γ, LIMP-II, tyrosinase CD4, and GLUT4 have a [DE]XXXL type signal, whereas a DXXLL signal has been characterized in mannose 6-phosphate/insulin-like growth factor-II receptor, the cation-dependent mannose-6-phosphate receptor, LDL-receptor related proteins, β-secretase, and others (Bonifacino and Traub, 2003).

We have shown that PSMA undergoes internalization via clathrin-coated pits in LNCaP cells (Liu *et al.*, 1998), a human prostate cancer cell line that abundantly expresses endogenous PSMA. To further understand the mechanism of PSMA internalization, we searched for possible internalization motifs in the cytoplasmic domain of PSMA. In this study we demonstrate that the cytoplasmic tail N-terminal five amino acid residues, including the start codon methionine and the fifth amino acid residue leucine form a novel MXXXL motif that mediates PSMA internalization. By transferring this MXXXL motif to a noninternalized protein, the α-chain of interleukin 2-receptor (Tac), we further show that this motif is sufficient for PSMA internalization. Furthermore, using an inducible dominant-negative μ2 subunit of the adaptor protein (AP)-2 adaptor complex, we provide evidence that internalization of this clinically important protein requires the function of AP-2.

## EXPERIMENTAL PROCEDURES

### *Plasmid Constructs*

Cloning and characterization of full-length cDNA of PSMA was described previously (Israeli *et al.*, 1993). The alanine scan mutagenesis approach was used to mutate each of the cytoplasmic tail amino acids in the cytoplasmic tail of PSMA. Alanine scan mutagenesis was essentially carried out by polymerase chain reaction (PCR) by using sense primers carrying respective mutation of the cytoplasmic tail amino acid (positions 2–15) to alanine. A Kozak consensus sequence (GCCACC) and a translation start site (ATG) were incorporated at the N-terminus of the sense primers. A cytoplasmic tail deletion mutant of PSMA was created by deleting the N-terminal 15 amino acids by using PCR. An alanine and three arginine residues proximal to the transmembrane domain were retained because these three arginine residues may be necessary to maintain the type II orientation of the protein (von Heijne, 1988). Also, PSMA constructs in which the cytoplasmic tail amino acids 6–14 were

| Construct | Cytoplasmic Tail Sequence | | | | | | | | | | | | | | | | | | | | Int |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **PSMA** | | | | | | | | | | | | | | | | | | | | | |
| Wt | M | W | N | L | L | H | E | T | D | S | A | V | A | T | A | R | R | P | R | | + |
| Δcd | M | → | | | | | | | | | | | | | A | R | R | P | R | | - |
| Ala 4,5 | - | - | - | A | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | | - |
| Ala 4 | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | | + |
| Ala 5 | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | | - |
| Ala 2 | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | | + |
| Ala 3 | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | | + |
| Ala 6 | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | | + |
| Ala 7 | - | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | | + |
| Ala 8 | - | - | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | | + |
| Ala 9 | - | - | - | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | | + |
| Ala 10 | - | - | - | - | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | | + |
| Val 14 | - | - | - | - | - | - | - | - | - | - | - | - | - | V | - | - | - | - | - | | + |
| Ala 8,10,14 | - | - | - | - | - | - | - | A | - | A | - | - | - | A | - | - | - | - | - | | + |
| Δ6-14 | M | W | N | L | L | → | | | | | | | | | A | R | R | P | R | | + |
| MA(5) | M | W | N | L | L | H | E | T | D | S | A | V | A | T | A | R | R | P | R | | - |
| | A | A | A | A | A | | | | | | | | | | | | | | | | |
| **Tac** | | | | | | | | | | | | | | | | | | | | | |
| Tac Wt | R | R | Q | R | K | S | R | R | T | I | | | | | | | | | | | - |
| Tac-MWNLL | - | - | - | - | - | - | - | - | - | - | L | L | N | W | M | | | | | | + |
| Tac-MWNAA | - | - | - | - | - | - | - | - | - | - | A | A | N | W | M | | | | | | - |
| Tac-MWNAL | - | - | - | - | - | - | - | - | - | - | L | A | N | W | M | | | | | | + |
| Tac-MWNLA | - | - | - | - | - | - | - | - | - | - | A | L | N | W | M | | | | | | - |
| Tac-AWNAL | - | - | - | - | - | - | - | - | - | - | L | A | N | W | A | | | | | | - |
| Tac-MAWNAL | - | - | - | - | - | - | - | - | - | - | L | A | N | W | A | M | | | | | - |

**Figure 1.** Schematic representation of PSMA cytoplasmic tail mutants and Tac-PSMA chimera used in this study. Deletions are shown by horizontal arrows, and insertion of additional alanines is indicated. Amino acids converted to alanine or valine are indicated as A and V, respectively. Internalization (INT) positive (+) or negative (−) for the respective constructs is indicated.

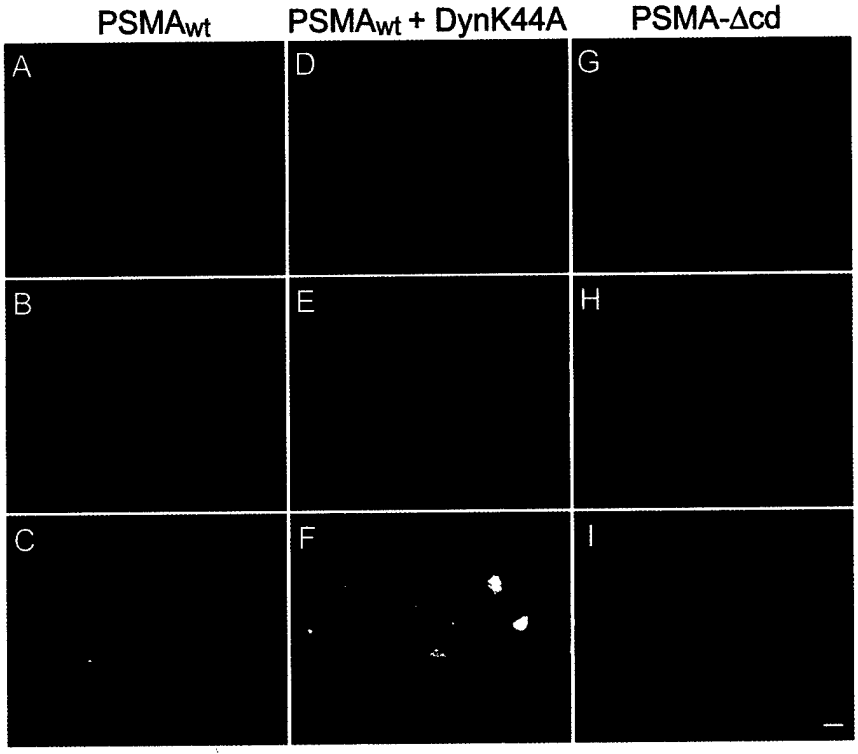


**Figure 2.** PSMA internalization in COS-7 cells expressing wild-type PSMA ($PSMA_{wt}$) and the cytoplasmic tail deletion mutant (PSMA-Δcd). (A–C) Internalization of $PSMA_{wt}$ and FITC-transferrin. COS cells transiently transfected with $PSMA_{wt}$ were simultaneously incubated with mAb J591 (A) and FITC-transferrin (B) for 2 h, washed, fixed in cold methanol, and stained with Texas Red-conjugated anti-mouse antibody. Representative medial optical sections are shown. (C) Merged image. The yellow color indicates the codistribution of FITC-transferrin and internalized PSMA. (D–F) COS cells expressing Dynamin K44A and $PSMA_{wt}$ cDNA were incubated with mAb J591 for 2 h, washed fixed, and stained with FITC-conjugated anti-mouse antibody to detect PSMA (D) and with polyclonal anti-dynamin antibody and Texas Red-conjugated anti-rabbit antibody to detect cells expressing the dynamin mutant (E). (F) Merged image. Note that in cells expressing DynaminK44A, PSMA was not internalized. (G–I) PSMA-Δcd–expressing cells were incubated with mAb J591 (G) and FITC-transferrin (H) as described above. PSMA-Δcd does not internalize and therefore, does not colocalize with internalized transferring (I). Bar, 5μm.

deleted or all the three putative phosphorylation sites were mutated (PSMA-T8A/S10A/T14A) and a PSMA construct containing five alanines inserted after the start codon [PSMA-MA(5)] were generated using PCR. Tac-PSMA chimera were also generated using PCR. Full-length Tac (gift from Dr. Bonifacino, National Institutes of Health, Bethesda, MD) was described previously (Leonard *et al.*, 1984). Tac cytoplasmic tail chimera containing the di-leucine–like motif of PSMA (Tac-MWNLL), di-leucine motif mutated to alanine (Tac-MWNAA), leucine at position 5 mutated to alanine (Tac-MWNLA), leucine at position 4 mutated to alanine (Tac-MWNAL), methionine at first position mutated to alanine in Tac with leucine at position 4 mutated to alanine (Tac-AWNAL), and with an extraalanine (Tac-MAWNAL) were generated. Because Tac is a type I membrane protein, to have the N-terminal methionine free as in PSMA, we used primers encoding the respective amino acids in the reverse orientation. Full-length PSMA (designated as wild-type PSMA [$PSMA_{wt}$]), cytoplasmic tail mutants of PSMA, and Tac-PSMA chimeras were inserted into eukaryotic expression vector pCDNA3. The mutations were verified by DNA sequencing. Constructs used in this study are shown in Figure 1.

### *Cell Culture and Transfection*

COS-7 cells (ATCC CRL 1651) were grown in DMEM supplemented with 10% fetal bovine serum containing streptomycin and penicillin at 5% $CO_2$ in a water-saturated atmosphere. Cells grown on glass coverslips were transiently transfected by the calcium phosphate method as described previously (Rajasekaran *et al.*, 1994). After transfection (48 h), the cells were tested for the uptake of antibodies as described below. HeLa cells expressing hemagglutinin-tagged D176A/W421A mutant μ2 constructs under the control of a tetracycline-repressible promoter have been described previously (Nesterov *et al.*, 1999). The cells were grown in DMEM supplemented with 10% fetal bovine serum containing streptomycin and penicillin, 400 μg/ml G418, 200 ng/ml puromycin, and 10 ng/ml doxycycline at 5% $CO_2$ in a water-saturated atmosphere. Cells plated on glass coverslips were used for transient transfection by the calcium phosphate method. Twelve hours after transfection, expression of the mutant μ2 protein was induced by replacing the culture medium with doxycyline-free medium. Eight hours before the planned experiments, sodium butyrate was added to the culture medium to ensure high expression levels of the mutant μ2 protein to replace the endogenous wild-type μ2 in AP complexes. Transfected cells were used 60 h after transfection.

### *Antibody Uptake and Immunofluorescence Analysis*

Antibody uptake was carried out as described previously (Liu *et al.*, 1998). In brief, the cells were washed with DMEM containing 0.5% fatty acid-free bovine serum albumin and incubated at 37°C for 2 h with mAb J591 (5 μg/ml). Cells were then fixed, permeabilized, and incubated with Texas Red-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). To visualize PSMA localization in endosomes, cells were coincubated with fluorescein isothiocyanate (FITC)-conjugated transferrin (Jackson ImmunoResearch Laboratories) during J591 incubation. To monitor the internalization of Tac-PSMA chimera, mAb against the extracellular domain of Tac, 7G7 (Rubin *et al.*, 1985) was used. For kinetic analysis of PSMA uptake the cells were incubated with J591 and FITC-conjugated transferrin for 1 h at 4°C, washed three times, and then incubated in DMEM at 37°C, 5% $CO_2$ to allow for uptake. The cells were fixed at the indicated time points and incubated with Texas Red-conjugated secondary antibody. Uptake of antibodies (mAbs J591 and 7G7) and transferrin were visualized and quantitated by confocal microscopy (see below). To visualize surface expression of PSMA and Tac-PSMA chimeras, COS cells transfected with the respective plasmid were fixed and stained with mAb J591 and 7G7, respectively, under nonpermeabilized conditions.

### *Confocal Microscopy*

Codistribution of internalized mAbs J591or 7G7 and transferrin were examined using a Fluoview laser scanning confocal microscope (Olympus America, Melville, NY). To detect simultaneously FITC- and Texas Red-labeled antigens, samples were excited at 488 and 568 nm with argon and krypton lasers, respectively, and the light emitted between 525 and 540 nm was recorded for FITC and >630 nm for Texas Red. Images were generated using Fluoview software (version 2.1.39). Transfected cells (30–40) were examined for each transfection done in duplicate and the representative data are shown.

Quantification of internalization in COS cells expressing $PSMA_{wt}$ and PSMA harboring mutation of the fourth leucine (PSMA-L4A) or fourth and fifth leucine (PSMA-L4A/L5A) was done using image analysis software (Fluoview, version 2.1.39). Average pixel intensities of internalized transferrin (green), and mAb J591 (red) from optical sections of 30–40 cells were determined. Because the transferrin uptake was more or less uniform PSMA internalization was normalized to transferrin uptake. An analysis of variance was used to compare the PSMA/transferrin ratios as a function of time between $PSMA_{wt}$ and PSMA-L4A. A logarithmic transform was used to stabilize variance and for computing 95% confidence intervals for the geometric mean of PSMA-L4A mutant ratios as a percentage of $PSMA_{wt}$ ratios.

### *NAALDase Activity*

NAALDase activity was determined as described by Sekiguchi *et al.* (1989). COS cells were transfected with $PSMA_{wt}$ on 60-mm culture dishes. After 48 h of transfection, cells were incubated with 1 μCi/ml [$^3$H]NAAG (PerkinElmer Life Sciences, Boston, MA) in Krebs-Ringer bicarbonate medium or in Dul-

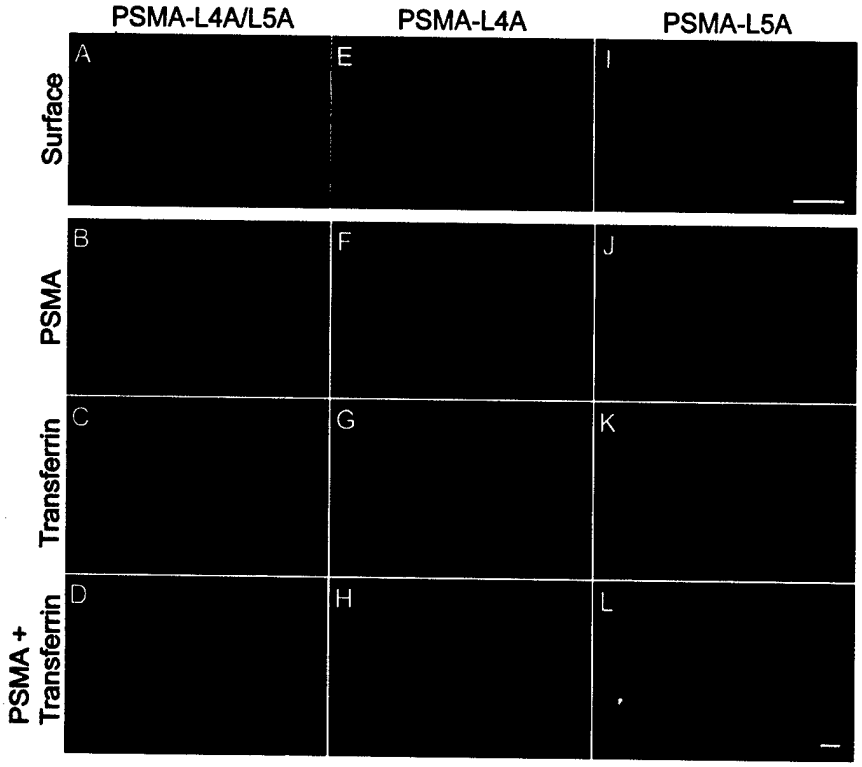


**Figure 3.** Internalization of the cytoplasmic tail di-leucine mutants of PSMA. (A, E, and I) Surface expression of PSMA in COS-7 cells expressing PSMA-L4A/L5A, PSMA-L4A, and PSMA-L5A mutants, respectively. Forty-eight hours after transfection, the cells were fixed in paraformaldehyde under nonpermeabilized conditions and labeled with mAb J591 followed by FITC-conjugated anti-mouse antibody and visualized by epifluorescence microscopy. (B, F, and J) Internalization of PSMA mutants. (C, G, and K) FITC-transferrin uptake. (D, H, and L) Merged images of PSMA and FITC-transferrin. Representative medial optical sections are shown. Yellow color in H indicates the codistribution of FITC-transferrin and internalized PSMA. Bars, 10 $\mu$m (A, E, and I) and 5 $\mu$m (B, C, D, F, G, H, K, and L).

becco's modified Eagle's medium for 1 h. The medium was removed and the cells were washed three times with their respective incubation medium. Cells were then lysed in 1% Triton X-100, and the radioactivity was determined using a scintillation counter (Beckman LS 6500). Counts were normalized to protein. Protein concentrations of the cell lysates were determined using the Bio-Rad DC reagent (Bio-Rad, Hercules, CA) according to manufacturer's instructions.

## RESULTS

To study the internalization of PSMA, COS cells were transiently transfected with $PSMA_{wt}$ cDNA (Figure 1) and uptake of mAb J591 was monitored by immunofluorescence and confocal microscopy. The internalized antibody showed a distinct spot-like staining pattern at the perinuclear region (Figure 2A). This spot-like staining is reminiscent of the recycling endosomal compartment and internalized transferrin, a marker for this compartment, colocalized with endocytosed PSMA (Figure 2, A–C), indicating that PSMA is localized to the recycling endosome. We have shown earlier that PSMA is internalized via clathrin-coated vesicles in LNCaP cells (Liu *et al.*, 1998). To further confirm that PSMA is internalized via a clathrin-dependent endocytic mechanism in COS cells, we tested whether PSMA is internalized in cells expressing a GTPase-deficient dynamin mutant (K44A), which is known to inhibit clathrin-dependent endocytosis in cultured cells (Herskovits *et al.*, 1993; van der Bliek *et al.*, 1993). In these cells internalization of PSMA was not detected (Figure 2, D–F) further confirming that PSMA is internalized via a clathrin-dependent endocytic pathway.

To test whether the cytoplasmic tail of PSMA contains a signal that mediates its internalization the PSMA cytoplasmic tail was deleted and the mutant (PSMA-Δcd) was expressed in COS cells. PSMA-Δcd was clearly expressed on the cell surface as revealed by immunofluorescence staining under nonpermeabilized condition (our unpublished data). Incubation of these cells with mAb J591 did not show uptake or localization to the endosomes and internalized transferrin did not reveal a codistribution with PSMA (Figure 2, G–I), indicating that the cytoplasmic tail of PSMA contains a signal that mediates its internalization.

The cytoplasmic tail of PSMA contains two consecutive leucines as reported for di-leucine-like motifs (Figure 1). To examine whether this motif functions as an internalization signal for PSMA, the di-leucine pair was converted to di-alanine (PSMA-L4A/L5A), the mutant protein was expressed in COS cells and uptake of mAb J591 was monitored. The di-alanine mutant of PSMA was clearly expressed on the cell surface as revealed by staining with mAb J591 under nonpermeabilized condition (Figure 3A). Our internalization assay revealed that mAb J591 was not internalized in cells expressing the di-alanine mutant of PSMA (Figure 3B) and did not show codistribution with the internalized FITC-transferrin (Figure 3, C and D), indicating that mutation of the di-leucine pair in the cytoplasmic tail of PSMA abrogates its internalization. We then examined whether both these leucines are essential for the internalization of PSMA. For this purpose single leucine residues at positions 4 (PSMA-L4A) and 5 (PSMA-L5A) were mutated to alanine and the uptake of mAb J591 was studied. Both these mutants were expressed on the cell surface as revealed by staining with mAb J591 under nonpermeabilized condition (Figure 3, E and I). J591 was clearly internalized in cells expressing PSMA-L4A (Figure 3F) and internalized transferrin (Figure 3G) codistributed with the internalized mAb J591 (Figure 3H). By contrast, in cells expressing PSMA-L5A, mAb J591 was not internalized (Figure 3J) and the antibody primarily stained the plasma membrane similar to cells expressing PSMA-L4/L5 (Figure 3B). In these cells, colocalization of PSMA and internalized FITC-transferrin was not

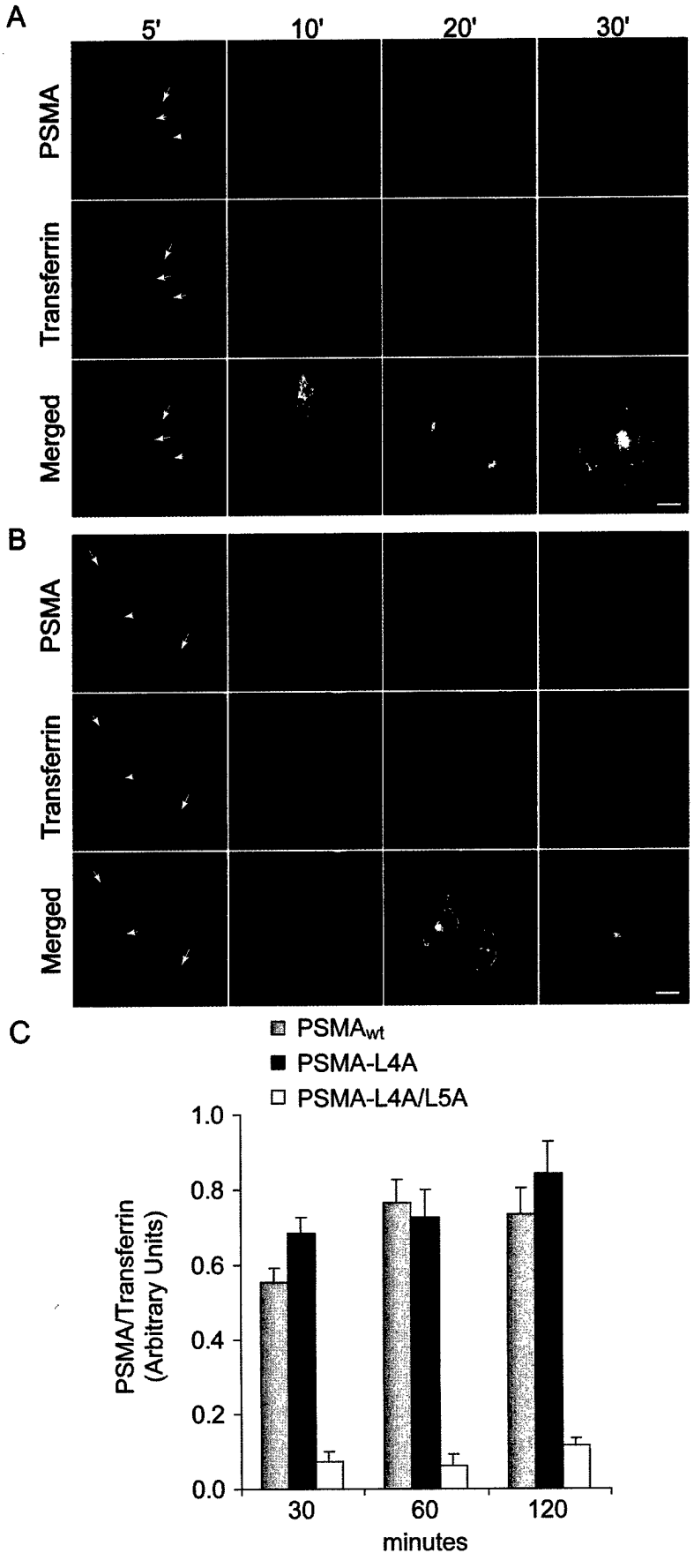


**Figure 4.** Kinetic analysis of internalization of PSMA$_{wt}$ and PSMA-L4A in COS cells. (A and B) Time course of PSMA$_{wt}$ (A) and PSMA-L4A (B) internalization. Transiently transfected COS cells were incubated with mAb J591 and FITC-transferrin for the indicated time points, as described under EXPERIMENTAL PROCEDURES and stained with Texas Red-conjugated anti-mouse antibody. Representative medial optical sections are shown. Arrows indicate peripheral vesicles containing PSMA and transferrin. Bar, 5 $\mu$m. (C) COS cells expressing PSMA$_{wt}$, PSMA-L4A, or PSMA-L4A/L5A were incubated with J591 and FITC-conjugated transferrin for 1 h at 4°C, washed, and incubated at 37°C to allow for uptake. The cells were fixed after 30, 60, and 120 min and incubated with Texas Red-conjugated secondary antibody. Uptake of mAbs J591 and transferrin were visualized and quantitated by confocal microscopy as described under EXPERIMENTAL PROCEDURES. PSMA internalization was normalized to transferring uptake. The bars indicate SE of 30–40 cells analyzed for each condition.

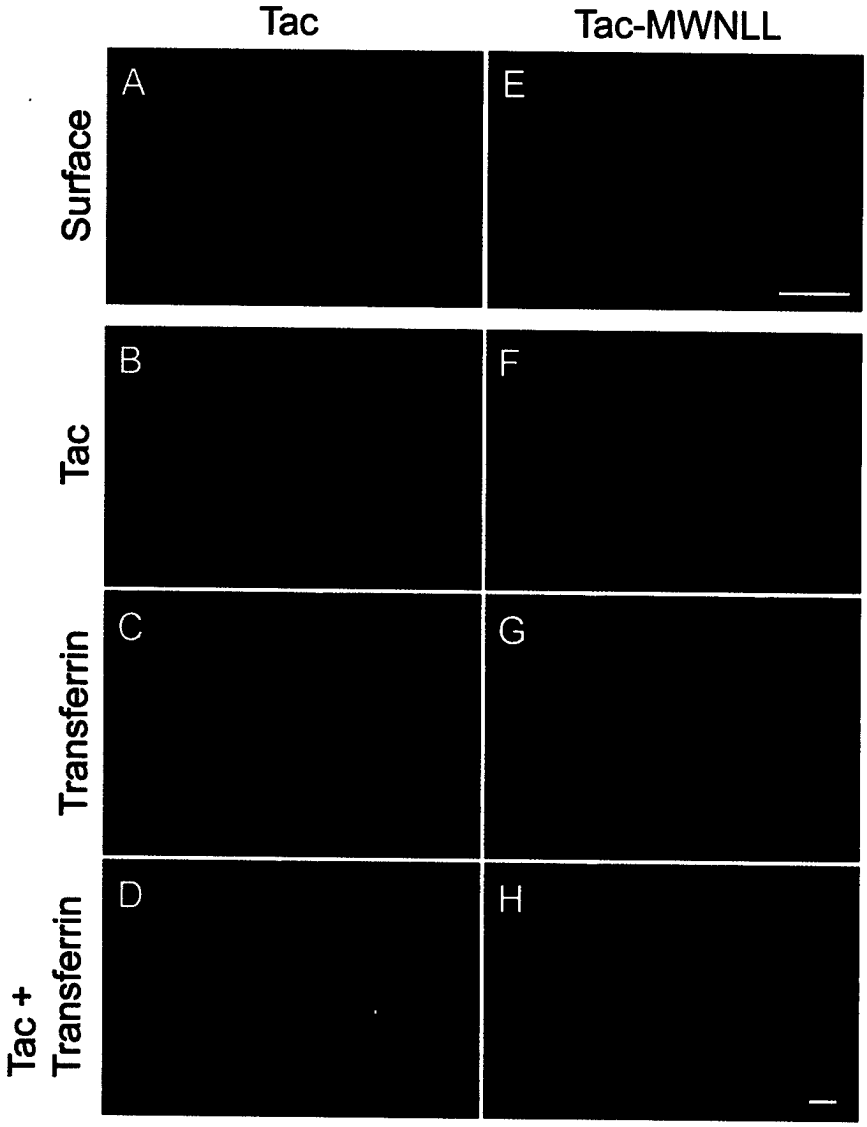


**Figure 5.** Internalization of Tac and Tac-PSMA chimera. (A and E) Surface expression of Tac. Forty-eight hours after transfection the cells were fixed in paraformaldehyde under nonpermeabilized condition, labeled with mAb 7G7 followed by FITC-conjugated anti-mouse antibody, and visualized by epifluorescence microscopy. (B–D, F–H) Internalization of Tac and FITC-transferrin. The cells were incubated with mAb 7G7 and FITC-transferrin for 2 h, washed, fixed in cold methanol, and stained with Texas Red-conjugated anti-mouse antibody. Representative medial optical sections are shown. (B and F) Internalization of Tac antibody. (C and G) Uptake of FITC-transferrin. (D and H) Merged images. Yellow color in H indicates the codistribution of FITC-transferrin and internalized Tac. Bars, 10 $\mu$m (A and E) and 5 $\mu$m (B, C, D, F, G, and H).

detected (Figure 3, K and L). These results indicated that the fifth leucine in the cytoplasmic tail of PSMA is crucial for its internalization.

Because PSMA-L4A was internalized similar to $PSMA_{wt}$ we determined the kinetics of PSMA uptake in cells expressing these constructs. Our efforts to obtain quantitative data by using iodinated mAb J591 were not successful because mAb J591 bound to the cell surface was not quantitatively released after acid wash procedures commonly used to release bound antibody on the cell surface. Therefore, we used immunofluorescence and confocal microscopy approaches to determine the kinetics of mAb J591 uptake in cells expressing $PSMA_{wt}$ and PSMA-L4A. As shown in Figure 4, both $PSMA_{wt}$ and PSMA-L4A expressing cells internalized PSMA rapidly. After 5-min incubation both PSMA and transferrin showed predominant plasma membrane localization with small amounts localized to peripheral vesicles (arrow). Similarly, after 10 min PSMA and transferrin codistributed in more peripheral vesicles, whereas after 20 min it accumulated in the recycling endosomal compartment. Cells expressing PSMA-L4A (Figure 4B) showed a similar internalization pattern. These results indicated that mutation of the fourth leucine in the cytoplasmic tail of PSMA has a minimal effect on the internalization of PSMA in COS cells. To obtain quantitative data we determined the average pixel intensity represented by internalized PSMA and transferrin by using image analysis software (Fluoview, version 2.1.39). Because quantification of internalized PSMA and transferrin was more reliable after 30 min, we quantified internalized PSMA in cells expressing $PSMA_{wt}$ and PSMA-L4A at 30, 60, and 120 min. We used internalized transferrin as an internal control for defining the area representing the internalized PSMA. Comparison of the internalization kinetics of $PSMA_{wt}$ and PSMA-L4A revealed that PSMA-L4A is internalized with kinetics similar to $PSMA_{wt}$ (Figure 4C). An analysis of the variance demonstrated that internalization increased with time ($p = 0.04$), but there was no statistical difference between the internalization profiles for $PSMA_{wt}$ and PSMA-L4A mutants ($p > 0.2$). The 95% confidence intervals for PSMA-L4A mutant internalization (as percentage of $PSMA_{wt}$) were 100–148% at 30 min, 72–116% at 60 min, and 89–143% at 120 min, indicating that mutation of the fourth leucine does not alter the internalization properties of PSMA.

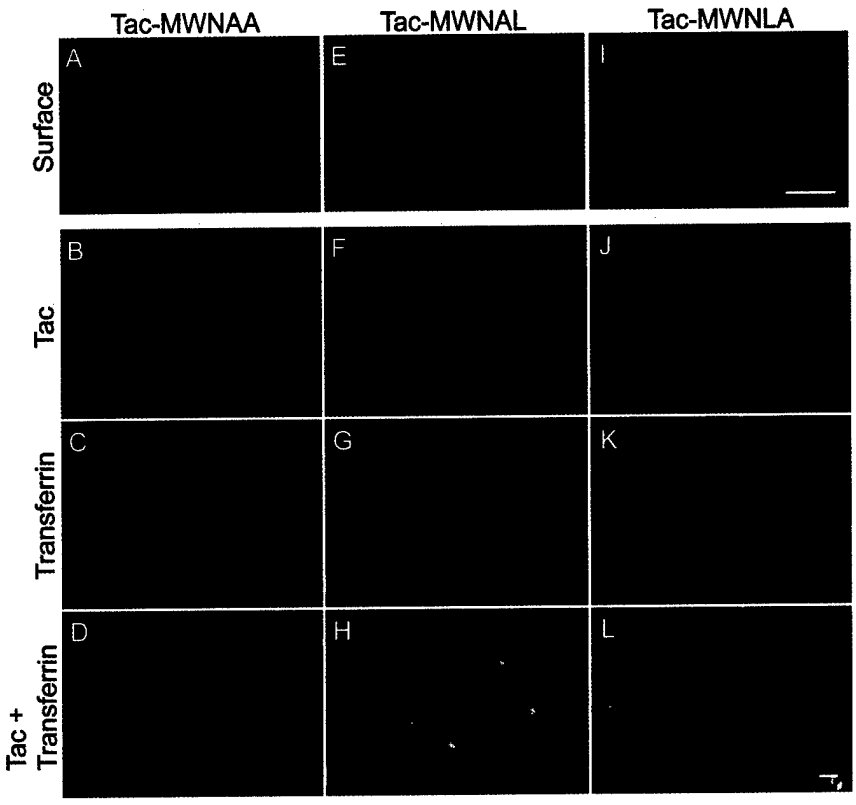


**Figure 6.** Internalization of Tac-PSMA chimeras harboring mutations in the di-leucine signal. Surface expression as well as internalization of PSMA was performed as described in figure legend 5. (A, E, and I) Surface expression of Tac in COS-7 cells expressing Tac-MWNAA, Tac-MWNAL, and Tac-MWNLA chimeras, respectively. (B, F, and J) Internalization of Tac chimera mutants. (C, G, and K) Uptake of FITC-transferrin. (D, H, and L) Merged images. Representative medial optical sections are shown. Yellow color in H indicates the codistribution of FITC-transferrin and internalized PSMA. Bars, 10 $\mu$m (A, E, and I) and 5 $\mu$m (B, C, D, F, G, H, J, K, and L).

To further test whether amino acid residues other than the fifth leucine are essential for the internalization of mAb J591 we systematically mutated each of the cytoplasmic tail amino acids into alanine. These point mutations did not affect the internalization of mAb J591 (our unpublished data). Moreover, the construct in which amino acids 6–14 were deleted (PSMA-Δ6–14) internalized mAb J591 when expressed in COS cells. These results demonstrated that the N-terminal first five amino acids in the cytoplasmic tail of PSMA are sufficient to mediate PSMA internalization and the fifth amino acid leucine is crucial for its internalization activity.

To further confirm that this five amino acid motif of PSMA is sufficient for internalization, we transferred the five N-terminal amino acids of PSMA to the noninternalized protein Tac, a type I membrane protein (Letourneur and Klausner, 1992). Internalization of Tac was monitored by uptake of mAb 7G7 raised against the extracellular domain of Tac (Rubin *et al.*, 1985). In nonpermeabilized COS cells wild-type Tac ($Tac_{wt}$) showed a distinct plasma membrane localization (Figure 5A), indicating that this protein is targeted to the plasma membrane but incubation with mAb 7G7 did not result in the internalization of this antibody, confirming that $Tac_{wt}$ is not internalized in COS cells (Figure 5B) as reported previously (Letourneur and Klausner, 1992). Codistribution of mAb 7G7 staining and internalized transferrin was not detected in these cells (Figure 5, C and D). By contrast, incorporation of the amino acids MWNLL into the Tac cytoplasmic tail (Tac-MWNLL) resulted in the internalization of mAb 7G7 (Figure 5F). The internalized antibody clearly colocalized with internalized FITC-transferrin (Figure 5, G and H) indicating that the N-terminal five amino acids in the cytoplasmic tail of PSMA are transferable and are sufficient to confer internalization properties to a noninternalized protein.

Furthermore, in cells expressing Tac-MWNAA where the two consecutive leucines are mutated to alanines, the mAb 7G7 was not internalized (Figure 6B), although this protein was clearly localized to the plasma membrane (Figure 6A). This mutant did not codistribute with internalized FITC-transferrin (Figure 6, C and D). Internalization of mAb 7G7 was maintained in cells expressing the construct where the fourth leucine is mutated to alanine (Tac-MWNAL) (Figure 6, F–H), whereas in cells expressing Tac-MWNLA, where the leucine at position 5 is mutated, the uptake of 7G7 was not detected (Figure 6, J–L). Both these mutants were clearly expressed on the plasma membrane as revealed by nonpermeablized staining by using mAb 7G7 (Figure 6, E and I). Together, these data demonstrate that the leucine at the fifth position is critical for PSMA internalization.

Although it is possible that a single leucine in the cytoplasmic tail of PSMA might play a crucial role in its internalization, it is unlikely that it can function as an internalization motif. Therefore, we decided to test for other potential amino acid residues in the five amino acid motif that might be involved in the internalization of PSMA. We have evidence that mutation of amino acids at position 2 and 3 (our unpublished data) and 4 (Figure 3) of the cytoplasmic tail of PSMA did not affect internalization, whereas mutation of leucine at position 5 abolished its internalization. The only amino acid that remained to be tested was the first amino acid methionine. Therefore, we mutated methionine in the internalizing Tac-MWNAL chimera to generate Tac-AWNAL. Although Tac-AWNAL was expressed on the cell surface (our unpublished data), a drastic reduction in the internalization of mAb 7G7 was noticed in cells expressing

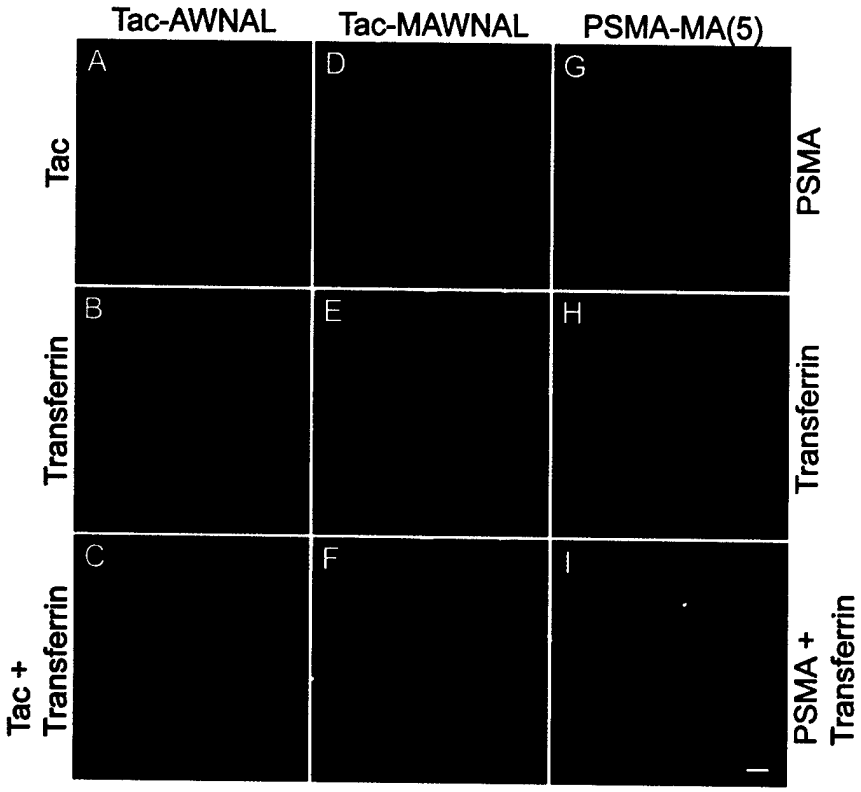


**Figure 7.** Internalization of Tac-PSMA chimeras Tac-AWNAL and Tac-MAWNAL, and of PSMA-MA(5). (A and D) Internalization of Tac chimera mutants and (G) mAb J591 in transiently transfected COS cells. (B, E, and H) Internalization of FITC-transferrin. (C, F, and I) Merged images. Note the lack of codistribution of Tac-chimera mutants or PSMA-MA(5) and FITC-transferrin. Bar, 5 μm.

this chimera (Figure 7A). Although in these cells FITC-transferrin was clearly internalized (Figure 7B), there was little colocalization of internalized transferrin with Tac-AWNAL (Figure 7C). Small amounts of internalized mAb J591 were seen in peripheral vesicles, in contrast to the intense fluorescence of internalized transferrin seen at the cell center. This result indicated that in addition to the fifth leucine the methionine is also required and that the N-terminal five amino acids, MWNLL, form a motif to mediate the internalization of PSMA. To test whether the length of this motif is involved in PSMA internalization, we inserted an additional alanine between tryptophan and methionine (Tac-MAWNAL) and monitored the internalization of this chimera. In COS cells, Tac-MAWNAL was clearly expressed on the cell surface as revealed by nonpermeabilized staining (our unpublished data). However, internalization of mAb7G7 was highly reduced (Figure 7D), and there was less colocalization of the chimera with internalized FITC-transferrin (Figure 7, E and F). We then tested whether incorporation of alanine into the MWNLL motif of PSMA itself affects internalization. Whereas insertion of one or two amino acids did not affect internalization, insertion of five alanines [PSMA-MA(5)] drastically reduced the internalization of PSMA (Figure 7G).

The endocytic motif of membrane receptors binds to AP complexes, which are heterotetramers and mediate the internalization of membrane receptors (Hirst and Robinson, 1998; Kirchhausen, 1999). The adaptor complex AP-2 has been shown to associate with both tyrosine (Ohno *et al.*, 1995; Honing *et al.*, 1996; Boll *et al.*, 2002) and di-leucine–based signals (Hofmann *et al.*, 1999). To obtain insights into whether AP-2 is involved in the internalization of PSMA, we monitored internalization of PSMA in a HeLa cell line that expresses a dominant negative mutant μ2 of the AP-2 complex under the control of a tetracycline-off system (Nesterov *et al.*, 1999). Strikingly, mutant μ2 drastically reduced the internalization of PSMA (Figure 8A) and transferrin (Figure 8B), and transferrin showed a more diffused localization pattern that codistributed with PSMA (Figure 8C). In non-induced cells that only express wild-type μ2 PSMA as well as transferrin were clearly internalized (Figure 8, D–F), indicating that the μ2-subunit of AP-2 is involved in the internalization mediated by the MWNLL motif of PSMA.

## DISCUSSION

In this study, we demonstrate that the cytoplasmic tail five N-terminal amino acids MWNLL are sufficient to mediate the internalization of PSMA. Methionine at the first position and leucine at the fifth position are essential, whereas amino acids 2, 3, and 4 are dispensable for the internalization of PSMA. Incorporation of alanine(s) into Tac-chimera (Tac-MAWNAL) and into PSMA [PSMA-MA(5)] drastically reduced the internalization, indicating that the length of this sequence is also important for its internalization function. We also present evidence that the adaptor complex AP-2 is involved in the internalization of PSMA. Based on these results, we suggest that the N-terminal five amino acid residues of PSMA form a novel autonomous methionine-leucine based internalization motif (MXXXL). To our knowledge, this is the first study describing a N-terminal amino acid (translation start site) as part of an internalization motif.

Although the presence of two consecutive leucines at position four and five suggested that the cytoplasmic tail of PSMA may contain a di-leucine–like motif, our results indicate that this might not represent a typical di-leucine motif as observed in other membrane proteins. The di-leucine–based signals of the [DE]XXXL[LI] and DXXLL types have an acidic residue at −4 from the first leucine (Pond *et al.*,

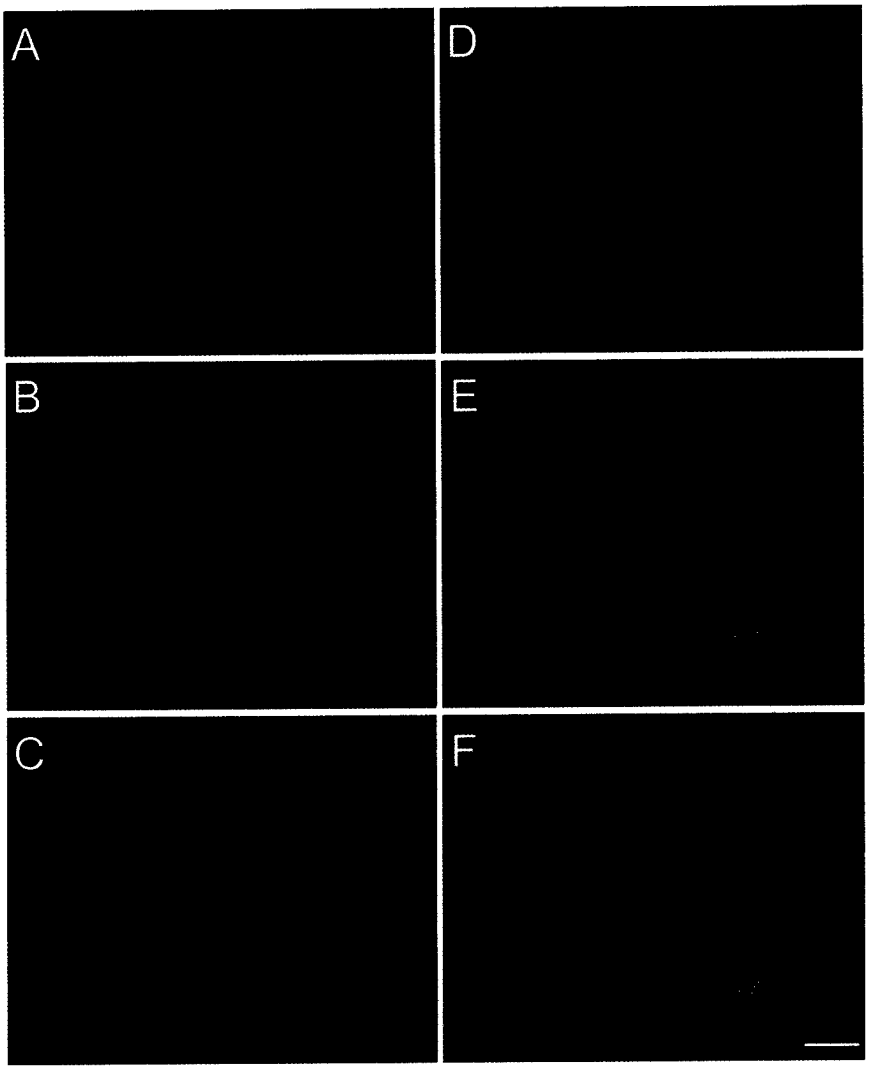


**Figure 8.** Internalization of $PSMA_{wt}$ in HeLa cells expressing dominant-negative AP-2 complexes. $PSMA_{wt}$ cDNA was transiently transfected into HeLa cells expressing a tetracycline-repressible dominant-negative mutant of $\mu$2. mAb J591 internalization was monitored in mutant $\mu$2-induced cells (A) and in noninduced cells (D). (B and E) Internalization of FITC-transferrin. (C and F) Merged images. Bar, 5 $\mu$m.

1995; Sandoval *et al.*, 2000), which is absent in PSMA and is replaced by an essential methionine. In the [DE]XXXL[LI] type the first leucine is generally indispensable and substitution with other amino acids decreases the efficacy of the signal (Letourneur and Klausner, 1992), whereas in the DXXLL type both the leucines are essential and mutation of any of these residues to alanine inactivates the signal (Johnson and Kornfeld, 1992; Chen *et al.*, 1997). In PSMA, mutation of the first leucine did not change significantly the internalization kinetics. Moreover, in polarized epithelial cells, proteins with di-leucine motif are targeted to the basolateral plasma membrane (Sheikh and Isacke, 1996; El Nemer *et al.*, 1999; Bello *et al.*, 2001). By contrast, PSMA is targeted to the apical plasma membrane in Madin-Darby canine kidney cells (Christiansen *et al.*, 2003) and swapping the cytoplasmic tail of PSMA with the cytoplasmic tail of a di-leucine motif containing protein redirected PSMA to the basolateral plasma membrane (our unpublished data). The absence of tyrosine residues in the cytoplasmic tail of PSMA clearly indicates that this protein does not contain a tyrosine-based signal. Together, these results strongly indicate that the MXXXL motif of PSMA is a novel methionine-leucine–based internalization motif.

PSMA is localized to the recycling endosomal compartment as revealed by its colocalization with internalized transferrin. Colocalization of Tac-MWNLL with transferrin further indicated that the MWNLL sequence is sufficient for the localization of PSMA to the recycling endosomal compartment. We have recently shown that the cytoplasmic tail of PSMA associates with the actin cross linking protein filamin (Anilkumar *et al.*, 2003) and that this association is involved in the localization of PSMA to the recycling endosomal compartment. Future studies will determine whether the MWNLL motif of PSMA associates with filamin and functions as a recycling endosome targeting signal.

We have shown that dominant negative $\mu$2 of the AP-2 complex reduces the internalization of PSMA, indicating that the AP-2 complex is involved in the internalization mediated by the MXXXL motif of PSMA. Recent structural studies suggested that the YxxΦ endocytic determinant might associate with the $\mu$2 adaptin as a two-pinned plug into a socket with the Y and the Φ residues (the pins) fitting into sterically and chemically complementary pockets of the $\mu$2 surface (Owen and Evans, 1998; Owen and Luzio, 2000). Requirement of the specific length of the MXXXL motif tempts us to speculate that the first amino acid methionine

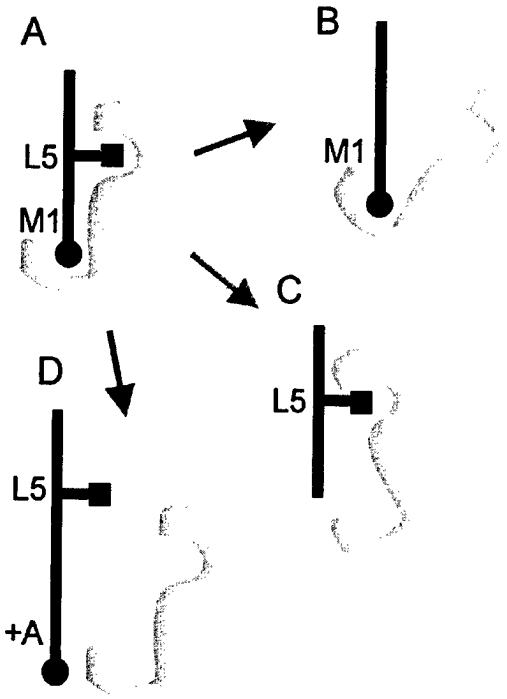


**Figure 9.** Schematic model of binding of the PSMA internalization motif to $\mu$2 of the AP-2 complex. (A) The endocytic determinant of PSMA might form two pins (methionine at position 1 [black circle] and leucine at position 5 [black square]) that fit into a complementary pocket of a $\mu$2 (gray) associating with the cytoplasmic tail of PSMA. Loss of the side chains of leucine-5 (B) or methionine-1 (C) of the internalization motif might result in an altered association of the adaptor preventing the internalization of PSMA. Similarly, extension of the length of the internalization motif with an additional alanine (D) might prevent the binding of the adaptor protein to the cytoplasmic tail of PSMA and therefore inhibit internalization of the protein.

and the fifth leucine of the PSMA endocytic determinant might function as two pins fitting into a complementary pocket of $\mu$2 (Figure 9). Future studies will establish whether $\mu$2 directly associates with the MXXXL motif and whether this motif can compete with the YxxΦ or [DE]XXXL[LI] motifs, which are known to associate with $\mu$2.

The catalytic site for glutamate carboxypeptidase/NAALDase activity of PSMA resides in its extracellular domain (Speno *et al.*, 1999). Millimolar concentrations of phosphate used in the culture medium almost completely inhibited the NAALDase activity in COS cells (our unpublished data; Slusher *et al.*, 1999). Because our internalization assays were performed in culture medium that inhibits NAALDase activity, this enzymatic activity seems not to be necessary for the internalization of PSMA. Moreover, in LNCaP cells, incubation with the NAAG substrate for NAALDase did not increase the internalization of PSMA (our unpublished data), whereas incubation with mAb J591 or the Fab fragments of this antibody increased the internalization rate of PSMA (Liu *et al.*, 1998). The antibody and the antibody fragments might mimic a putative ligand for PSMA. These results indicate that the internalization of PSMA might be an independent function from its glutamate carboxypeptidase/NAALDase activity. It is possible that PSMA might function as a receptor mediating the internalization of a putative ligand. Identification of a PSMA ligand combined with the knowledge on PSMA internalization should provide more insights into the function of this protein and, in consequence, provide valuable information for therapeutic applications of PSMA.

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